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Early grain filling environment and pre-maturity α -amylase formation in UK winter wheat genotypes

Andro F.J. Tjin-Wong-Joe MSc.

A thesis submitted to the Open University for the degree of
Doctor of Philosophy

September 2004



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Studiare è inutile tutte le idee si affollano su te

Laura Pausini

Abstract

When the UK winter wheat variety Rialto produced unexpected low Hagberg falling numbers that could not be directly linked to sprouting, it was proposed that these reductions in quality could be due to PMAA. The problem was not identified during the selection and commercial development stages.

In this project the hypothesis was tested that the variety Rialto is PMAA-susceptible. In addition, the effect of a previously proposed high temperature stimulus for PMAA followed by slow grain drying was assessed on plants grown in a controlled environment, throughout. This was done to eliminate any distorting effects the temperature history of plants grown in field or glasshouse had in previous studies. The identification of an effective and reliable PMAA stimulus could facilitate the development of a screening method for PMAA.

Analysis was done on 13 year-location combinations of field grown Rialto. Together, visual and chemical assessments of sprouting and iso-electric focusing of α -amylase isozymes identified several samples with significant α -amylase activity in the apparent absence of sprouting. In addition, tests with α -amylase sensitive Phadebas gel revealed distinctive PMAA discoloration patterns in 10% to 44% of the grain from the 13 samples, leading to the conclusion that Rialto is PMAA-susceptible. Results from a controlled environment experiment confirmed the ability of a high temperature treatment to induce increased amylase production in the grain. However, the increase occurred in low humidity, considered to promote grain drying. Other results suggested that induction transpires during an earlier grain development stage than proposed in previous studies. Further work revealed the potential of a detached tiller technique to be used in a prospective fast and reliable screening method for PMAA.

Declaration of Authorship

This thesis has been written by myself and describes my own work. It has not previously been submitted for a degree or other qualification at the Open University or any other educational establishment.

Andro F.J. Tjin-Wong-Joe

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Publications

Tjin Wong Joe, A.F., Summers, R.W., Lunn, G.D., Atkinson, M.D. and Kettlewell, P.S. (200..) Pre-maturity α -amylase and incipient sprouting in UK winter wheat, with special reference to the variety Rialto. *In Press**

Tjin Wong Joe, A.F., Simon, L., Summers, R.W., Flatman, E.A., Lunn, G.D. and Kettlewell, P.S. (2001) Does the wheat cultivar Rialto produce pre-maturity alpha-amylase? *Aspects of Applied Biology*, **64**, 163-164. (AAB symposium Poster Abstract, not refereed)

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Abbreviations

°Cdays	degrees(Celsius)days	(is a measurement for plant development)
ΔT	diurnal temperature difference	
ABA	abscisic acid	
AFRC	Agricultural and Food Research Council	
AMY	α-amylase isozyme	
ANOVA	analysis of variance	
CCFRA	Campden and Chorleywood Food Research Association	
cDNA	complementary DNA	(also popularly known as 'copy DNA')
CE	controlled environment	
CE HA	controlled environment experiment at Harper Adams	
CE M	controlled environment experiment at Monsanto Cambridge	
cGMP	cyclic GMP	
cv.	cultivar	
CV	coefficient of variation	
DE	detached ear	
df	degrees of freedom	
DH	double haploid	
DNA	deoxyribonucleic acid	
DT	detached tiller	
dw	dry weight	
ELISA	enzyme-linked immunosorbent assay	
<i>et al.</i>	<i>et alii</i>	(Latin meaning 'and others')
EU	enzyme unit	
FDB	fluorescein dibutyrates	
FRET	fluorescence resonance energy transfer	
fw	fresh weight	
GA	gibberellic acid	
GABP	GARE-binding protein	
GARE	gibberellin response element	
GMP	guanosine monophosphate	
GNB	gibbestatin B	
HFN	Hagberg falling number	
HGCA	Home Grown Cereals Authority	
IAA	indoleacetic acid	
IEF	iso-electric focussing	
LEA	late-embryogenesis abundant	
LMA	late maturity α-amylase (activity)	
L.S.D.	least significant difference of means at 5% level	
mRNA	messenger RNA	

MS	Murashige and Skoog
NIAB	National Institute of Agricultural Botany
NIL	near-isogenic line
NMR	nuclear magnetic resonance
OGT	O-linked N-acetylglucosamine transferase
OU	Open University
P	probability
PAR	photosynthetically active radiation
PBI	Plant Breeding International (was formerly Plant Breeding Institute)
PBIC	PBI Cambridge
PDA	potato dextrose agar
PEG	polyethylene glycol
pI	iso-electric point
PMAA	pre-maturity α -amylase activity
PoMS	post-maturity sprouting
PrMS	pre-maturity sprouting
QTL	quantitative trait locus
rcf	relative centrifugal force
RH	relative humidity
Rht (rht)	reduced height
RNA	ribonucleic acid
RPAA	retained pericarp α -amylase activity
r.p.m.	revolutions per minute
s.d.	standard deviation
S.E.M.	standard error of means
sp.	species
spp.	species (plural)
TIF	tagged image file
T _{max}	maximum daily temperature
T _{min}	minimum daily temperature
UK	United Kingdom
UV	ultraviolet
ZGS	Zadoks' growth stage

Due to the large number and changing nomenclature of genes and sub-sequences, most abbreviation used to name genes (in *italic*) and their protein products (capitals), used once or only sporadically in the text, are not included in the list above.

1 General introduction

The winter wheat variety Rialto has been a successful variety listed from 1995, for over half a decade, on the UK recommended list of winter wheats. Developed by PBI Cambridge it continued to be marketed under the PBIC Seeds label after Unilever sold Plant Breeding International (PBI) to Monsanto in 1998, to become the main site in the UK for Monsanto (UK) Ltd.'s plant breeding operations. Classified as a group 2 winter wheat, Rialto has less breadmaking potential than varieties listed in group 1, a list of established varieties favoured for breadmaking. Rialto has however also performed less consistently compared to other varieties within the group 2 category, for instance compared to PBI's newer variety Option. What surprised the wheat breeders most was that some Rialto crops would produce low breadmaking quality grain when this was not expected. This tendency of Rialto to yield sporadic and inexplicable poor quality only become apparent after the variety had gone through breeding and commercial evaluation processes and was placed on the recommended list of varieties. Termed 'Rialto syndrome' it was clear to plant breeders at PBI that the selection methods were not effective in screening out this specific problem.

It was a meeting between Dr Richard Summers from Monsanto (UK) Ltd. and Dr Peter Kettlewell from Harper Adams University College during a seminar by Dr Kettlewell, that lead to the inception of this PhD research project in 2000. Dr Bernard Major, a former PhD research student of Dr Kettlewell at Harper Adams, had looked at the problem of pre-maturity α -amylase activity (PMAA) in winter wheat. This was done as part of a large collaborative project investigating the various routes of α -amylase accumulation in wheat grain. The project carried out in the mid-1990s was funded by the Home Grown Cereals Authority (HGCA) and their counterpart in France. An excess of α -amylase will reduce the

breadmaking potential of wheat flour. This reduction in quality occurs in most instances as a result of sprouting. Dr Kettlewell suggested that 'Rialto syndrome' however might be due to PMAA. Co-ordination of the various studies into α -amylase accumulation during the HGCA project lay with Dr Gavin Lunn from the University of Nottingham, Sutton Bonington. Dr Lunn was approached once more to act as second technical supervisor during this project. Sprouting and PMAA are two of several possible routes of grain α -amylase accumulation, which are discussed in the next chapter.

The immediate aim was to identify PMAA in grain samples of cv. Rialto, supplied by Monsanto (UK) Ltd. With PMAA shown to be present in Rialto grain, the notion that pre-maturity α -amylase is involved in the sporadic and unexpected deterioration of breadmaking quality in Rialto could henceforth be contemplated. Building on work done earlier by Dr Bernard Major, the effect of different environmental factors on PMAA expression was examined, not only for Rialto but also for a range of other genotypes. Experiments were carried out in controlled environment (CE) cabinets at Harper Adams University College and in larger CE rooms at Monsanto Cambridge. The ambitious goal of developing a breeders' screen for PMAA was not reached during this project, but an encouraging start was made with work on a detached tiller (DT) method for UK winter wheat genotypes, regarded as an essential step towards the implementation of a commercially viable PMAA screen.

2.1 Introduction

The presence of excessive α -amylase activity in bread flours has been taken to be a cause of poor bread-making quality. In fact, of the many grist products bread-making and noodle flour are particularly sensitive to high enzyme levels as processing conditions are favourable to enzyme catalysed product degradation (Evers *et al.*, 1999). The major effect of α -amylase action is due to the production of high molecular weight starch degradation products or dextrins (Gold and Duffus, 1996). The subsequent reduction in viscosity (deMan, 1999) lowers Hagberg Falling Number (HFN) (Hagberg, 1960), the most widely used test for α -amylase activity in the UK and Europe. Sprouting is the most important source of native α -amylase in wheat flour (Lunn *et al.*, 2001a and 2001b). It was not until the 1980s that retention of pre-mature α -amylase in wheat grain was also recognised as a potential problem in breadmaking, although Bingham and Whitmore (1966) did report the existence of pre-mature α -amylase almost one and a half decades earlier. Up until then most research and breeding efforts had focussed on sprouting resistance in breadmaking varieties. Nowadays pre-harvest sprouting, although originally intended as a term for germination of ripe grain in the ear before harvest, has become a general description of any cause of excessive native α -amylase in breadmaking grists (Gale and Lenton, 1987). The progress that has been made over several decades of research on the mechanism of pre-maturity α -amylase formation in wheat grain, is the main focus of the following literature review. However, in order to make for a more comprehensive reading, appropriate aspects of cereal grain morphology and physiology will be reviewed first.

2.2 Cereal grain morphology and physiology

Cereals are cultivated species of grasses that grow throughout the temperate and tropical regions of the world and as members of the Poaceae, or grass family, share similar features that are developed to different degrees in the various members (Evers *et al.*, 1999).

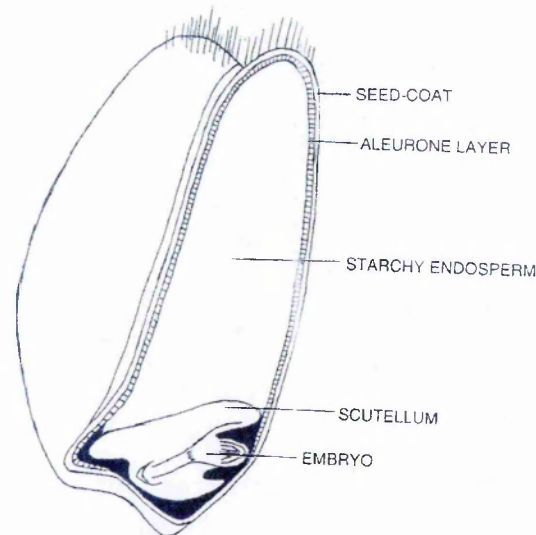


Figure 2.1 A longitudinal section through the crease region of a *Triticum* grain (redrawn from an illustration in Esau (1953))

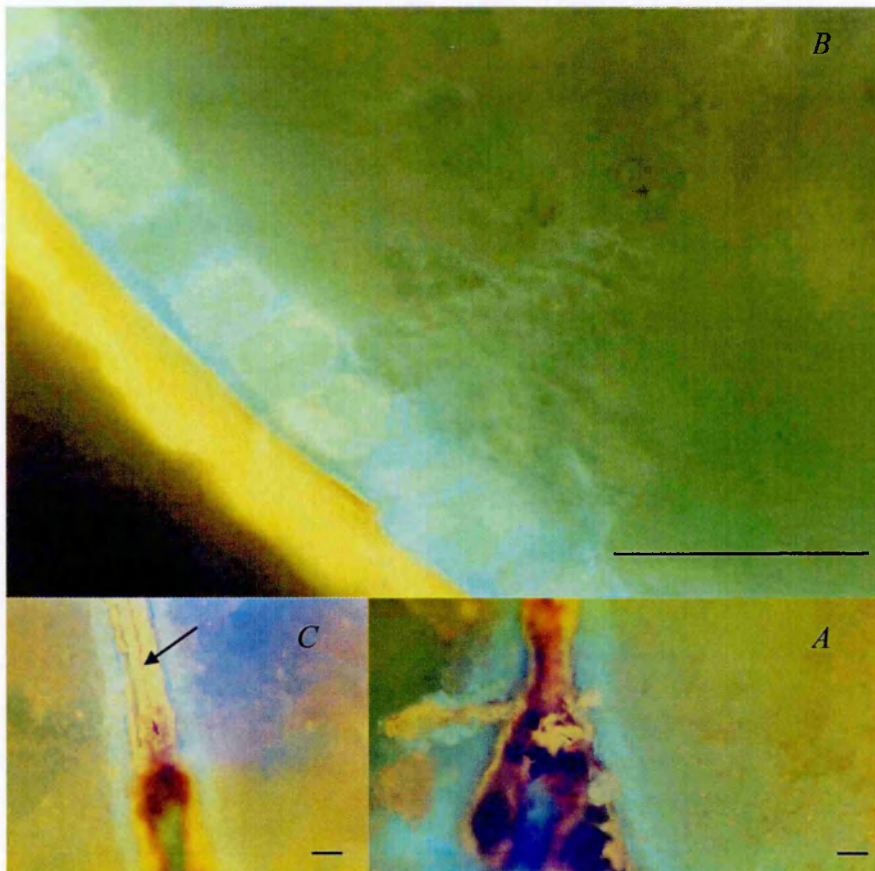
As the basic structure of cereal grains is surprisingly consistent (Evers *et al.*, 1999) wheat grain will be used as a model for cereal grain morphology and physiology. A grain of wheat, often called seed, is a nut-like fruit termed by botanists, a caryopsis (Percival, 1974). It contains a single seed or kernel enclosed within a thin shell. The seed, however, instead of being free as in many nuts, is adherent to the inner wall of the pericarp or shell, and the two cannot be separated readily. Grains of all wheat varieties have the same fundamental structure and consist of a seed-coat or testa (sometimes termed spermoderm), the embryo (which will later grow into a young wheat plant), the nucellar layer (in ripe grain not more than a layer of crushed cells between the testa and underlying layers) and the endosperm (starchy or floury part of the seed). In Gramineae, as in the other monocots, the single cotyledon (the main site of food reserve accumulation in dicots) is much reduced

and modified to form the scutellum (Bewley and Black, 1994). The endosperm consists mainly of thin-walled parenchymatous tissue stored with food for the nutrition of the embryo when germination commences and may be divided into two parts, the so-called aleurone layer and the starch- and gluten-parenchyma (Percival, 1974) (see Figure 2.1). In cereals the majority of cells in the endosperm are nonliving at maturity, the cytoplasmic contents having been occluded by the stored reserves during development. On the outside of the endosperm however there remains living tissue, the aleurone layer. This thin layer of cells does not store many reserves (Bewley and Black, 1994), but is involved in the release of enzymes for their mobilisation. The aleurone layer is almost entirely one cell thick and closely follows the contours of the seed-coat. Only in the crease, and occasionally at other parts, are two or more cells superposed (Percival, 1974) (Plate 2.1 *A* and *B*).

A common characteristic of developing seeds is that they do not contain active reserve-mobilising enzymes, which are normally produced or are activated only when seeds have germinated (Garcia-Maya *et al.*, 1990). A notable feature in the biology of seeds is the temporal separation of developmental and germinative events (Cornford *et al.*, 1987a). Embryogenesis and embryo growth occur during seed development but the further conversion into a seedling normally only proceeds when mature seeds, frequently after rehydration, germinate. Development of the cereal grain can be divided into two stages, grain enlargement and grain filling (Bewley and Black, 1994). Enlargement is the result of cell division followed by an influx of water which drives cell extension. Filling occurs as the reserves, starch and protein, are deposited within the endosperm. In fact, the major metabolic activity during development is associated with the synthesis and deposition of seed reserves, while during the germinative phase, this is replaced by the production of a variety of mobilising enzymes followed by reserve mobilisation (Bewley and Black, 1994).

Plate 2.1 Fluorescence image of transverse sections through a wheat grain, with the aleurone blue and the pericarp yellow fluorescent.

A. Multi-layered crease aleurone, *B.* Single cell aleurone layer outside of crease region with more discernible individual cells, *C.* Collapsed crease cavity in mature grain (arrow)



Images taken at University of Nottingham, Sutton Bonington. Procedure supervised by Dr G.D. Lunn. Wheat aleurone morphology and fluorescence microscopy described in Greenwell *et al.* (2001). Bars correspond to 15 μm .

2.2.1 The α -amylase enzyme

α -Amylase is one of the primary hydrolytic enzymes in the grain. During cereal grain germination hydrolytic enzymes hydrolyse the endospermic reserves into a form which can be utilised by the growing embryo. In wheat grain the scutellum plays an important role in the initial hydrolysis of stored substrate during germination. However, although the scutellum is activated first during germination, the aleurone becomes more important to reserve mobilisation after a lag period (Ganguli and Sen-Mandi, 1993).

Starch is the major food reserve in the endosperm of cereal grain and the substrate for α -amylase action. Starch is a polymer of D-glucose and occurs as small granules in storage cells (deMan, 1999). It is composed of two different polymers, a linear compound, amylose, and a branched component, amylopectin (see Figure 2.2). In the linear fraction the glucose units are joined exclusively by α -1 \rightarrow 4 glucosidic bonds and the polymer chain takes the form of a helix. Amylopectin is branched because of the occurrence of α -1 \rightarrow 6 linkages at certain points in the molecule (deMan, 1999).

The amylases are the most important enzymes of the group of glycoside hydrolases. These starch-degrading enzymes can be divided into two groups, the so-called debranching enzymes that specifically hydrolyse the 1,6-linkages between chains, and the enzymes that split the 1,4-linkages between glucose units of the straight chains. The latter group consists of endoenzymes that cleave the bonds at random points along the chains and exoenzymes that cleave at specific points near the chain ends (deMan, 1999). α -Amylase, an endohydrolase, is important in the initial degradation of amylose and amylopectin and together with β -amylase, α -glucosidase (exohydrolases) and dextrinase, responsible for the reduction of starch into simple sugars (Jacobsen and Chandler, 1988). α -Amylase (α -1,4-glucan-4-glucanohydrolase) hydrolyses the α -1,4-glucosidic bonds in a random fashion along the chain. A mixture of amylose and amylopectin is hydrolysed into a mixture of dextrans, maltose, glucose, and oligosaccharides, thus reducing viscosity (deMan, 1999). During bread-making the hydrolysis of starch to simple sugars occurs rapidly in the baking process during the heating of the dough, particularly during the few minutes after starch gelatinisation at 60°C and before thermal inactivation of wheat α -amylase at 75°C (Gale and Lenton, 1987).

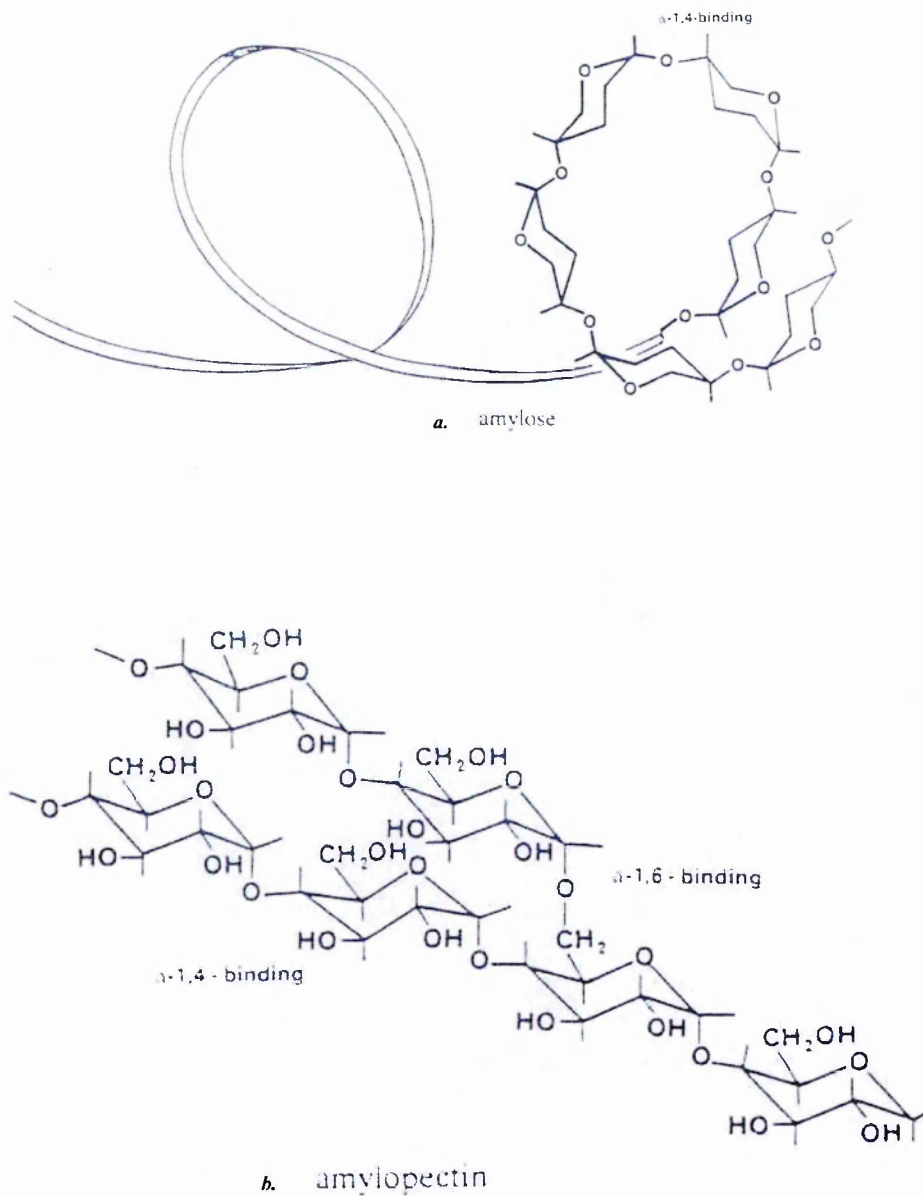


Figure 2.2 The molecular structure of starch; *a.* The amylose helix and *b.* The branched structure of amylopectin (from Engbersen and de Groot, 1995)

Bread wheat is an allo-hexaploid ($2n=6x=42$) (Li and Foley, 1997) with α -amylase loci on all three genomes, though each locus is actually composed of small multigene families (Flintham and Gale, 1988). Since the first demonstration that barley α -amylase could be resolved into a number of isozymes by electrophoresis, there have been many similar

reports of multiple forms of the enzyme from a number of cereals (Jacobsen and Chandler, 1988) including wheat.

The α -amylase isozymes separate into two iso-electric point (pI) groups. According to Mrva and Mares (1999a) isozymes of the high pI group in spring wheat have an iso-electric point between 6.3 and 7.5 and low pI isozymes have an iso-electric point between 4.9 and 6.0. Earlier electrophoretic studies (Olered and Jönsson, 1970) found wheat high pI isozymes to fall within the 6.0 to 6.5 range and low pI between 4.5 and 4.8. Isozymes in one group are more alike while there are characteristic differences between the two groups. In wheat, the high pI α -AMY1 (malt) isozymes are controlled by three α -Amy1 loci on the long arm of chromosome 6A, 6B and 6D (Gale and Lenton, 1983; Mrva and Mares, 1999a) and similarly α -Amy2 (low pI or green amylase) loci are located on the long arms of the group 7 chromosomes (Gale and Lenton, 1983). A third group of α -amylases, α -AMY3 (Baulcombe *et al.*, 1987), with a very high pI has recently been identified, but does not appear to be of great importance to total α -amylase activity.

2.2.2 Four mechanisms leading to α -amylase production in cereal grain

There are four known sources of α -amylase in wheat grain (Kettlewell, 1989), namely retained pericarp α -amylase activity (RPAA), pre-maturity α -amylase activity (PMAA), pre-maturity sprouting (PrMS) and post-maturity sprouting (PoMS). While PoMS, PrMS and PMAA are primarily caused by α -AMY1 isozymes initially secreted into the embryo-proximal endosperm area (PoMS and PrMS) or the crease region (PMAA) (Lunn *et al.*, 1996; Major, 1999), in RPAA grain enzyme activity is due to α -AMY2 isozymes (Lunn *et al.*, 1996). A similar problem to PMAA was described by Mrva and Mares (1996a) for

spring wheat in Australia and was termed late maturity α -amylase (LMA). These researchers did not report LMA to be more prevalent in the crease region.

The α -AMY2 isozymes in the pericarp are produced early in development and normally activity declines to undetectable levels as the pericarp matures, though this is not the case in grain affected by RPAA. PoMS is caused by dormancy break (next section) in grain with a moisture content high enough to allow the seeds to germination. In contrast, PrMS seems to be initiated before the onset of grain dormancy, though compared to PoMS relatively little is known about the phenomenon (Major, 1999). PMAA, the premature formation and retention of α -amylase in the endosperm without germination, is thought to occur under certain environmental conditions and with greater severity in more susceptible varieties.

An investigation into the four causes of α -amylase activity revealed a difference in occurrence between the four (Lunn *et al.*, 2001a). Of 56 variety-site-year combinations enzyme activity was detected in 32 cases. In 23 cases it was sufficiently high to cause a reduction of HFN, below the commercial criterion. The frequency of occurrence of the different modes of enzyme accumulation was found to be in the following order PoMS>PMAA>PrMS>RPAA (Lunn *et al.*, 2001a). Though both PMAA and PrMS were more common than expected, the most usual pattern was for α -amylase to accumulate by several modes. Lunn *et al.* (1996) had earlier demonstrated that it is possible to discriminate between the different types of α -amylase activity (chapter 3). They used iso-electric focussing to identify the α -amylase isozyme group produced and they identified incipient sprouting by visualising esterase/lipase activity in grain using the fluorescein dibutyrate (FDB) method (Jensen *et al.*, 1984).

2.2.3 Sprouting and dormancy

Seed dormancy is an important stage in the life cycle of many plants and is characterised by the temporary failure of a viable seed to sprout (germinate) under favourable conditions (Li and Foley, 1997). Dormancy promotes species survival by distributing germination in both time and space. In cereals where dormancy is weak, pre-harvest sprouting can occur under wet or humid conditions (Li and Foley, 1997), given that not only sufficient water but also oxygen is available at suitable temperatures (Kent and Evers, 1994). Breeders have over the ages striven to reduce the susceptibility to sprouting and red grain colour has traditionally been a marker for sprouting resistance in wheat breeding programs (Flintham, 2000). Dormancy however is not the only mechanism preventing sprouting in the grain. Dormancy is distinctly different from quiescence, the latter being a state of arrested development in non-dormant grains imposed by unfavourable environmental conditions (Figure 2.3).

Dormancy that develops at the end of grain maturation is called primary dormancy. Different factors regulate the degree of dormancy and they are located in various tissues of the grain. Dormancy can either be coat-imposed or be so-called true embryo dormancy (Li and Foley, 1997). A pleiotropic gene expressed in the testa influencing grain colour and associated with enhanced dormancy was first described by Biffen (1905) to have a Mendelian inheritance, with red grain dominant over white (Flintham, 2000). Pre-harvest sprouting still occurs sporadically in the UK even though all currently grown commercial varieties are red. This implies that the red-grained phenotype alone does not guarantee effective resistance (Flintham and Gale, 1988; Kettlewell *et al.*, 1999). In addition to testa based factors various genes acting in the embryo and affecting dormancy have been identified in different species (Flintham, 2000; Suzuki *et al.*, 2000; Holdsworth *et al.*,

2001). Induced dormancy in mature, partially or fully after-ripened (non-dormant) seeds is termed secondary dormancy (Li and Foley, 1997). It has been hypothesised that alterations in properties of cell membranes, brought about by changes in environmental conditions, are involved in the regulation of secondary dormancy (Hilhorst, 1998). The relationship between the non-dormant state and primary and secondary dormancy in seed is presented in Figure 2.3.

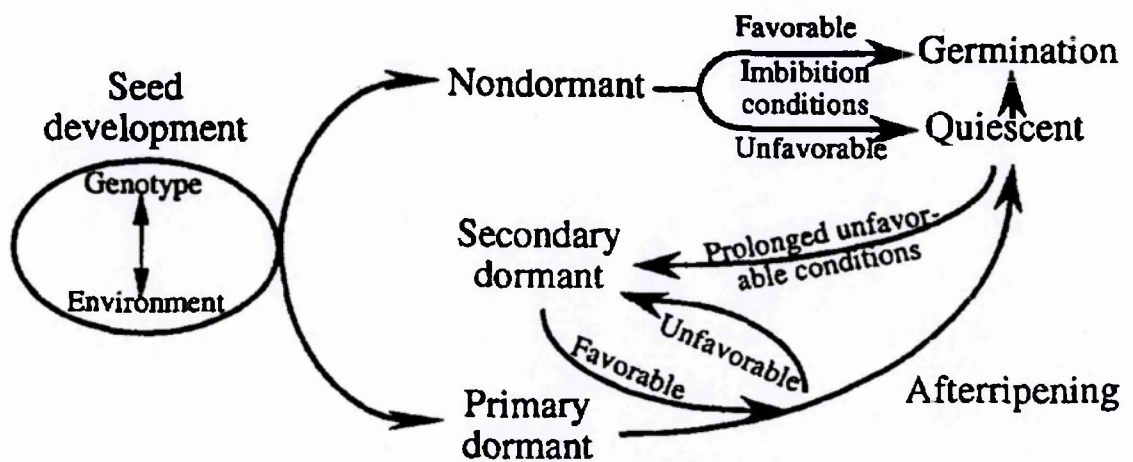


Figure 2.3 The relationship between non-dormant, primary dormant and secondary dormant states in seed (from Li and Foley (1997))

As grains mature a period of dormancy sets in, developing between one and two months after pollination (Kawakami *et al.*, 1997), with both environment and genotype influencing the length of the dormancy period. Although genes like the ones coding for red grain can enhance expression of dormancy, environmental conditions can on the other hand restrict the duration of the dormancy period thus increasing the chance of sprouting (Gale and Lenton, 1987). In general, a transition of many seeds from a dormant to a non-dormant state (after-ripening) is accomplished by exposing the seed for a period of time to specific environmental conditions (Li and Foley, 1997). After-ripening duration is regulated by the degree of dormancy and by environmental factors, with the degree of dormancy being influenced by genetic and environmental factors during seed development, and specifying

the germination phenotype. Environmental factors involved in dormancy break include temperature, moisture, oxygen and light. The type and levels required do not only differ between species, but in cultivated crops even vary between varieties.

A gene expressed in the seed-coat has been linked to varying levels of dormancy. The association of three homoeologous loci (located on chromosomes 3A, 3B and 3D) for red grain colour (*R-A1b*, *R-B1b* and *R-D1b* red phenotype/dominant and *R-A1a*, *R-B1a* and *R-D1a* white phenotype/recessive) with an extended dormancy phenotype has long been known and was first postulated by Nilsson-Ehle (1914), followed by numerous subsequent confirmations (Flintham, 2000). There is however great overlap between white and red wheats with regard to the level of dormancy expressed. According to Torada and Amano (2002), it is only under wet and humid conditions, for which most dormancy in white wheat is found to be inadequate, that a red seed-coat appears to convey sufficient levels of dormancy, essential to prevent germination under these conditions. After a review of the available literature Mares (1993) concluded that most previous studies were compatible with a model in which the expression of dormancy involves an interaction between seed-coat and wheat embryo. The latter exhibiting varying degrees of sensitivity depending on genotype, environmental conditions, and the stage of ripeness or after-ripeness. Mares (1993) proposed several potential factors involved in the interaction with the embryo which include ABA (Walker-Simmons, 1987; Morris *et al.*, 1989), catechins and catechin-tannins from the seed-coat and unidentified compounds from the bracts (Derera and Bhatt, 1980). Stoy and Sundin (1979) and Stoy and Olsen (1980) had earlier reported an association between sensitivity to germination inhibition by the possible precursors of the red seed-coat pigment, catechin-tannins.

Additional genetic effects that are independent of seed-coat pigmentation have also been implicated in the control of dormancy in white wheats (Anderson *et al.*, 1993; Mares, 1996). Paterson and Sorrells (1990) reported that dormancy in white kernelled wheat is a quantitative trait with dormancy most likely dominant. They also concluded from their experiments that the environment had a strong influence on the germination phenotype of white kernelled wheat and that dormancy heritability was low. In one of the crosses they made (dormant \times non-dormant) epistatic interactions were evident. It is thus safe to conclude that many (major and minor) genes influence expression of the trait. In fact, it has been observed that certain white genotypes express stronger dormancy than the weakest reds (Flintham, 1993). Although gene dosage effects might be an additional factor in variation between different red wheats, R gene dosage alone cannot account for the wide variation in dormancy between lines carrying all three dominant R alleles (Flintham, 1993). It has therefore been suggested that dormancy might not be a direct, pleiotropic effect of dominant R alleles, but rather that these genes are acting as linked markers (Stam and Zeven, 1981) for other dormancy genes (on the group 3 chromosome). Others have found evidence suggesting a pleiotropic association and phenotypic differences found in reciprocal F1's by Flintham (2000) could be accounted for by a direct effect of the maternal seed-coat pigment.

Flintham (2000) found that in certain crosses most of the difference in dormancy between the parental varieties is caused by a single gene for which he proposed to use the symbol *Phs* (pre-harvest sprouting). He found it unlikely that *Phs* operates via effects in the triploid endosperm and therefore prefers the hypothesis that *Phs* exerts its effects in the embryo. The *Phs* gene accounts for major variation in dormancy between different wheats with three dominant R alleles. Flintham (2000) finds it tempting to speculate that the zygotic mechanism might also operate in the absence of the red seed-coat pigment. Much

more has been learned about embryo based control of dormancy by detailed investigation of mutations affecting the trait. Some mutations in cereals affect seed development by causing vivipary (Li and Foley, 1997) which is germination prior to full maturation and possibly before shedding from the parent plant. Analysis of ABA-deficient mutants such as *viviparous* (*vp*) 2, 5, 7, 8 and 9 of maize and *aba1* of *Arabidopsis* indicates that ABA (section 2.2.4.2) in embryos and not in the endosperm, is critical for dormancy development (Karssen *et al.*, 1983). The sensitivity of embryos to ABA is another key factor in seed dormancy, since plants with mutations that render them insensitive to ABA such as maize *vp1* and *Arabidopsis abi1*, *abi2* and *abi3* exhibit viviparous germination or reduced dormancy (Koornneef *et al.*, 1989). In wheat the sensitivity of embryos to ABA is also closely correlated with the level of seed dormancy (Kawakami *et al.*, 1997). Suzuki *et al.* (2000) published results supporting the proposed involvement of ABA in the formation and maintenance of seed dormancy during middle and late wheat embryogenesis.

It has long been suggested that dormancy is associated with gene repression followed by de-repression as dormancy is broken (Tuan and Bonner, 1964), but only recently have genes been isolated and characterised that are differentially expressed in dormant and non-dormant grains. Prolonged synthesis of many heat-stable proteins (e.g. dehydrin) in axes of dormant wheat has been reported. In contrast, a few heat-stable proteins are synthesised in axes of non-dormant grains, but synthesis declines relatively fast compared to dormant grains (Ried and Walker-Simmons, 1990). Many genes expressed preferentially in dormant grains have been found to be responsive to ABA and analysis of sequence homology has indicated that most code for late-embryogenesis abundant (LEA) proteins (Li and Foley, 1997). It has been suggested that LEA proteins play a protective role during water loss (Dure, 1993), so it may be that these proteins function to maintain embryo viability during repeated cycles of desiccation in dormant grain, though it does therefore seem unlikely that

LEA proteins are involved in dormancy regulation. In fact, in their review paper Li and Foley (1997) conclude that there is as yet no direct candidate for involvement in the maintenance or termination of seed dormancy. More recently Holdsworth *et al.* (2001) reported that repression of germination by *ABI3* and other loci in *Arabidopsis* indicates that these factors interact with loci that enhance germination potential. They identified the *COMATOSE (CTS)* locus that regulates germination potential by enhancing after-ripening, sensitivity to gibberellins and pre-chilling, and by repressing the activities of loci that activate embryo maturation. Furthermore, they have analysed the structure and expression of wheat *Vp1* homeologues and have shown that both maturation and germination genes are expressed simultaneously in embryos grown under germination promoting conditions and that the majority of *VPI* transcripts expressed in the cytoplasm during normal embryo maturation are not correctly spliced. It can therefore be suggested that under perturbed environmental conditions wheat may not express enough functional VP1 activity to repress germination. As more evidence appears to show that embryogenesis and germination are under strict sequential and mutually exclusive control, a better understanding of the regulation of transition between these processes should provide important approaches to new technologies that can be used to improve grain quality traits (Holdsworth *et al.*, 2001). In addition to molecular analysis other research has also contributed to a better understanding of dormancy. Mares and Mrva (2001) found significant quantitative trait loci (QTL's) in Australian wheat, that explained 11%, 9% and 9% of the phenotypic variation and which were located on chromosome arms 2AL, 2DL and 4AL, respectively. They put forward that identification of QTL's offers the opportunity to develop molecular markers for grain dormancy and to develop a better understanding of the mechanisms involved in this trait.

2.2.4 Plant hormones in cereal grain

Endogenous growth regulators, or hormones, in developing seeds may be involved in several processes. Hormones can be involved in growth and development of the seed, including the arrest of growth prior to seed maturation. They can also affect the accumulation of storage reserves and the later release of these reserves, and bring about various physiological changes in tissues and organs close to the developing fruit (Bewley and Black, 1994). Bewley and Black (1994) listed the following hormones as having been isolated from immature wheat grains, auxin (IAA), gibberellic acids which include GA_{15,17,19,20,24,25,44,54,55} and 57, cytokinin (ribosyl zeatin) and abscisic acid (ABA), though absence of specific regulators does not mean that they do not exist in the seed, but rather that chemical characterisation has potentially not yet been made. The hormones GA and ABA are probably the two most important hormones in grain development and have a profound influence on α -amylase formation in cereal grain (Jacobsen and Chandler, 1988).

2.2.4.1 Gibberellic acid (GA)

Bioactive gibberellins affect a number of processes during plant development, including seed germination, leaf expansion, stem elongation, flower initiation and flower and fruit development (Sun, 2000). By the beginning of the 90's over 80 gibberellins were known, more than half of which had been found in developing seeds (Bewley and Black, 1994). GA synthesis follows the following route, acetyl coenzyme A \rightarrow mevalonate \rightarrow isopentenyl pyrophosphate \rightarrow chain elongation and ring closure \rightarrow *ent*-kaurene \rightarrow kaurenoic acid \rightarrow GA₁₂ aldehyde \rightarrow gibberellins. In general, developing seeds very actively interconvert GAs, which is why any one plant species contains several different kinds of GA in the immature seed. Some of the GAs and their catabolites may not be

biologically active, and hence, interconversion can lead to the biological inactivation of endogenous GA. However, at the early stages of seeds development the major GAs are biologically active and the inactive ones are formed towards the end of seed maturation (Bewley and Black, 1994). According to Gale and Lenton (1987) GA₁ is the primary 'germination-gibberellin' in wheat and it accumulates (to approximately 10 ng g⁻¹ fw) during grain germinative processes (Lenton *et al.*, 1987). Although GA₅₄ is present at high levels at maximum grain fresh weight (Gale *et al.*, 1987), the biological activity of GA₅₄ is some 30 to 100 fold less than that of GA₁ in inducing α -amylase production in mature, distal half grains and its concentration declines during grain maturation (Gale and Lenton, 1987). The various GAs may however be distributed unequally between grain tissues. In maize the GA₁ content of the embryo is about 40 times higher than in the endosperm (Bewley and Black, 1994). According to Bewley and Black (1994) one possible cause could be that GAs transported around the seed are subjected to differential metabolism at various stages.

2.2.4.2 Absciscic acid (ABA)

Although ABA is a relatively simple molecule, a mevalonic acid derived sesquiterpene, studies into its biosynthesis and physiological action have progressed slowly. The reason for this is, as is the case for other hormones, that in most plant tissues ABA levels are very low (4×10^{-8} M to 2×10^{-7} M) (Walton, 1988).

In a review of available literature Black (1991) concluded that ABA is one of the factors that constrains the embryo in its embryonic mode and at the same time arrests premature reserve mobilisation. For example, maize viviparous mutants with an ABA content of

6-16% of that of neighbouring wild-type kernels, do not dehydrate and germinate precociously, with neither endosperm produced nor maternally derived ABA able to prevent this (Gale and Lenton, 1987). In wheat, King (1976) has shown that when ABA levels are reduced by dehydration, precocious germination can occur. Similarly Quatrano (1986) demonstrated that removal of ABA from the culture medium induced immature excised embryos to germinate. The function of ABA in the developing grain appears to be twofold. ABA promotes storage protein synthesis (Bewley and Black, 1994) and prevents premature germination (Jacobsen and Chandler, 1988). In addition, ABA seems also to participate in the regulation of synthesis of several different kinds of proteins, which are thought to enable the maturing seed to withstand the rigors of desiccation (Black, 1991).

2.3 The gibberellin/ α -amylase system in cereal

Although most of the work on cereal aleurone response to gibberellin and abscisic acid has been done on barley, particularly with reference to α -amylase synthesis, it seems that many principles can also be applied to *in vivo* behaviour of other cereal grains (Jacobsen and Chandler, 1988). There are however differences in the pattern of GA accumulation during development and GA species present, between wheat and barley. For example, high pI α -amylase mRNA levels increase in response to exogenous GA, in mature barley scutellum (Chandler and Mosleth, 1990), while expression of high pI genes in mature wheat scutellum is independent of added GA₃ (Cejudo *et al.*, 1995).

The aleurone layer is the major site of hydrolytic enzyme activity within the grain (Ranki and Sopanen, 1984). However, in intact germinating wheat grain the initial phase of α -amylase synthesis takes place in the scutellum (Mrva and Mares, 1999a). The production of α -amylase by aleurone tissue is stimulated by GA and prevented by ABA mainly by

inhibition of gene expression or gene transcription (Jacobsen and Chandler, 1988).

Developing aleurone cells are normally incapable of responding to GA. Immature aleurone cells can be made sensitive to GA, in essence converted from the developmental to the germinative mode, by treatments such as drying or pre-incubation (Cornford *et al.*, 1986). During actual germination the high pI isozymes appear first, while this is the reverse for α -amylase produced in isolated barley aleurone (Jacobsen and Chandler, 1988). The emerging picture is of gibberellin perception at the plasma membrane, although GA-receptors have yet to be identified (Lovegrove and Hooley, 2000). GA₄ covalently coupled to membrane-impermeant Sepharose beads, stimulates high-level α -amylase gene expression and protein secretion in aleurone protoplasts. In addition, micro-injection of GA into aleurone protoplasts does not stimulate α -amylase gene expression and it is only when GA is present in the protoplast incubation medium, that a response is noticed. Studies have identified two GA-binding proteins that are found in the plasma membranes of the shoots and aleurone of several plant species (Lovegrove and Hooley, 2000; Sun, 2000). In contrast, other studies have detected soluble cytosolic or microsomal GA-binding proteins (Sun, 2000).

GA enhancement of α -amylase production increases with the time of exposure to the hormone (Ritchie *et al.*, 1999). Thus, once exposed to GA and after a certain lag period, the cells of the aleurone layer show detectable stimulation of α -amylase secretion. This GA-induced enhancement of α -amylase secretion with time is thought to be due to the recruitment of more and more cells to a secreting population rather than to a gradual increase in the secretory activity of all of the cells (Hillmer *et al.*, 1993). In addition to a time-dependent increase in secretion, GA also elicits the stimulation of secretion over a broad range of concentrations. Increasing GA concentration induces increasing α -amylase synthesis and secretion (Ritchie *et al.*, 1999). Observations made by Ritchie *et al.* (1999)

concur with the threshold model, originally developed by Rodbard (1973). Underlying this concept for a threshold basis of dose-response curves is a requirement for the cell-to-cell variation in hormone sensitivity that Ritchie *et al.* (1999) observed in both aleurone protoplasts and the intact aleurone layer. According to them, this ‘all-or-nothing’ recruitment model implies a hormonal response system in which GA throws a limited number of molecular ‘switches’ that engage the cellular machinery, leading to activated hydrolase synthesis and secretion. The basal state of GA signalling is likely to be repressive and the GA signal seems to activate the pathway by de-repression (Sun, 2000).

It has been shown that ABA reverses the stimulatory effect of GA on transcription of α -amylase genes in aleurone (Jacobsen and Chandler, 1988). Although ABA is known to regulate gene expression via an intracellular ABA receptor, the inhibitory effects of ABA on GA-signalling are most likely mediated through a receptor at the plasma membrane (Lovegrove and Hooley, 2000). Work by Mundy (1984) indicates that one of the mRNAs which accumulates in barley aleurone in response to ABA is for an endogenous inhibitor of the malt α -amylase 2 isozyme. Titration of mRNA with cDNA clones indicates that ABA blocks the synthesis of α -amylase at the transcriptional level while enhancing the synthesis of several proteins of unknown function in isolated barley aleurone layers (Mundy, 1984). An endogenous α -amylase inhibitor in barley has been shown to be synthesised shortly after fertilisation and to be most active during early seed development, with the protein becoming one of the more abundant proteins at maturity (Robertson and Hill, 1989). GA induces the production of large amounts of α -amylase and reduces synthesis of the α -amylase inhibitor. In contrast, synthesis and/or accumulation of the inhibitor is induced in embryoless barley half seeds incubated in ABA (Mundy, 1984). Hayashi *et al.* (2000) reported gibbestatin B (GNB), isolated from a *Streptomyces* species, to be an inhibitor of GA-induced expression of α -amylase in barley and rice. In addition to a GNB suppressed

accumulation of GA-induced barley high-pI type B and rice RamylA α -amylase they reported that GNB prevented GA-induced aleurone cell death. In tobacco and *Arabidopsis* GNB suppressed the germination and retarded the growth of seedlings without toxicity. The growth of certain GA-mutants (*gai*, *spy* and *abi* mutants) was also retarded. According to Hayashi *et al.* (2000), 'normal' plants treated with GA-biosynthesis inhibitors and GA-defective and GA-signalling mutants normally have dwarf dark green leaves. However, dwarfed healthy green leaves were observed in normal plants treated with GNB. They concluded that GNB inhibits the GA-induced expression of α -amylase by regulating one of the steps involved in ABA signalling.

Promoters of both low- and high-pI α -amylase genes contain a DNA sequence, the gibberellin response element (GARE), which is responsible for increased transcription in the presence of GA and suppression of transcription by ABA, but alone permits only very low levels of transcription from a promoter (Rogers *et al.*, 1996), probably because it offers a low affinity binding site for GARE-binding proteins (GABPs) (Lanahan *et al.*, 1992). DNA sequence elements flanking GARE in both low- and high-pI α -amylase promoters have effects on the level of transcription obtained, though the GARE function appears to be independent of these elements. Proteins binding to these (two) elements probably help position or stabilise binding of GABPs (Rogers *et al.*, 1996). Other elements have so far only been described in the low-pI promoters. Mutation of one, designated Box 5 (Rogers *et al.*, 1994) lowers the level of transcription to about 15% of maximum and mutation of another, designated O2S decreases transcription to a level only slightly above baseline (Lanahan *et al.*, 1992). In barley aleurone cells induction of transcription of the α -amylase genes by GA requires the GARE binding (Lovegrove and Hooley, 2000) transcription factor GAMYB (reviewed in Sun, 2000). Gubler *et al.* (1995) isolated the barley aleurone cDNA clone, *GAmYb*. *GAmYb* expression is up-regulated by GA and it has

been shown to activate transcription of a high pI α -amylase promotor in transient expression experiments. Gubler *et al.* (1995) postulated that the *GAmyb* is part of the GA-response pathway leading to α -amylase gene expression in aleurone cells. Another GARE binding factor, a zinc-finger transcriptional repressor termed HRT, has been found to inhibit GA-induced amylase expression (Lovegrove and Hooley, 2000; Sun, 2000). It has been suggested that the α -amylase appearing in the endosperm and aleurone of developing barley grains is produced in response to locally synthesised GA, since application of an inhibitor of GA synthesis to the developing grains reduces production of α -amylase (Duffus, 1969).

2.3.1 GA signalling

Most studies of GA receptors and GA-induced genes have focused on the cereal aleurone system (reviewed in Lovegrove and Hooley, 2000 & Sun, 2000). The expression of the genes encoding α -amylase, the most abundant hydrolase induced by GA in the aleurone layer, has been used as a valuable marker to monitor the status of the GA response in this tissue (Sun, 2000). In addition to studies on GA perception, the cereal aleurone system has also been used to identify protein components and second messengers that are involved in GA signalling, with candidates including heterotrimeric G proteins, Ca^{2+} , calmodulin (CaM), cGMP (cyclic GMP), and protein kinases (reviewed in Lovegrove and Hooley, 2000 & Sun, 2000).

In addition to biochemical studies another approach to examining GA signalling and response has been to identify mutations affecting these processes (Sun, 2000; Thornton *et al.*, 1999). GA-response mutants fall in to two phenotypic categories. First, mutants with the so-called 'GA-overdose' phenotype resemble wild-type plants that have been treated

with an excessive amount of GA. Second, the GA-unresponsive dwarf mutants fail to respond to exogenous GA treatment (Sun, 2000). Elongated (slender) mutants like the *sln* (slender) mutant in Barley and *spy* (spindly) and *rga* (repressor of *ga1-3*) mutants in *Arabidopsis* exhibit complete and partial GA-independent growth response respectively, and because of their recessive nature are likely to affect negative regulators of GA signalling. SPY is highly similar to the Serine/Threonine (Ser/Thr) *O*-linked N-acetylglucosamine (*O*-GlcNAc) transferases (OGTs) in rats and humans (Thornton *et al.*, 1999; reviewed in Sun, 2000). OGTs modify target proteins by glycosylation of Ser/Thr residues, which either interfere or compete with kinases for phosphorylation sites. Recessive GA-unresponsive dwarf mutants like the *dwarf1* mutant in rice, the *sleepy1* mutant in *Arabidopsis* and the *gse* mutants in barley have a semi-dwarf phenotype and may encode positive regulators of the GA signal transduction pathway. Finally, semi-dominant GA-unresponsive dwarf mutants like the *gai-1* (GA insensitive) mutant in *Arabidopsis* and *Rht1-3* mutants in wheat identify negative regulators in GA signalling (reviewed in Sun, 2000). It is hypothesised that the type of mutation in the *gai-1* protein makes it a constitutive repressor of GA response, whose activity cannot be inhibited by the GA signal. The semi-dominant GA-unresponsive dwarf mutants accumulate high levels of bioactive GA₁, suggesting that their impaired GA response upregulates GA biosynthesis via a feedback mechanism.

Sun (2000) presents a proposed role of the SPY, GAI and RGA proteins in the GA signalling pathway. A hypothetical transmembrane GA receptor is inactive in the absence of a GA signal. In this situation SPY is an active OGT and GlcNAc modifies RGA and GAI to their active state. Activated RGA and GAI function as activators or repressors of transcription and indirectly or directly inhibit the expression of GA-induced genes. When the hypothetical GA receptor is activated by binding of bioactive GA the GA signal

inhibits RGA and GAI not only by deactivating SPY, but also by an unidentified factor, which may deactivate RGA and GAI through interaction with the DELLA region. DELLA is a unique conserved region near the amino terminus and is called after a set of conserved amino acids (Thornton *et al.*, 1999; Sun, 2000). This region is thought to be involved in modulating GA response. The intensity of the signal coming from the GA-receptor complex will determine the level of SPY, RGA and GAI activity in a given cell. RGA and GAI may act as co-activators of repressors by interacting with other transcription factors because neither protein contains a well-defined DNA-binding domain (Sun, 2000).

2.3.2 α -Amylase in embryo and aleurone

Findings made by Cornford *et al.* (1987a) indicate that the predominant α -amylase protein produced by pre-mature excised wheat embryos is α -AMY2. Embryo/scutellar tissue of pre-mature wheat grains usually contain little α -amylase but readily produce the enzyme upon removal from caryopsis or surrounding tissue. The production of α -amylase is influenced by embryo age, stimulated by GA₃ and overall inhibited by ABA (Cornford *et al.*, 1987a). Working with immature excised embryos and using iso-electric focussing to identify α -amylase isozymes, Garcia-Maya *et al.* (1990) found that low-pI α -amylase mRNA transcripts are already present in developing wheat embryos (30 days post-anthesis), while high-pI isozymes are not. GA increases the amount of mRNA for the high-pI group but only slightly for the low-pI isozymes. In fact, Garcia-Maya *et al.* (1990) found evidence suggesting that the high-pI isozymes are dependent on endogenous GA *in situ*, while those from the low-pI group are not, although GA does have an influence on the pattern of low-pI isozymes expressed. In contrast, Cejudo *et al.* (1995) reported GA-independent high-pI gene expression in mature wheat scutellum. Osmoticum and ABA suppress the accumulation of high-pI isozymes, but do not affect the mRNA for the low pI

group (Garcia-Maya *et al.*, 1990), though 30 day old excised embryos incubated in ABA do produce an α -AMY2 isoenzyme (of relatively high pI) not found in non-ABA treated embryos (Cornford *et al.*, 1987a).

Cornford *et al.* (1987a) suggest that the regulatory mechanisms involving GA differ between scutellar (embryo) and aleurone cells. Results from experiments done by Mrva and Mares (1999a) also indicated that the control mechanism in scutellum of intact grains differs from that in isolated aleurone, though in germinated grain of the wheat variety Chinese Spring they found no difference in isozyme patterns between proximal (scutellum plus aleurone) and distal (aleurone only) halves. The α -amylase of a germinated mature grain and of aleurone tissue incubated in GA₃ is predominantly α -AMY1 (high pI) and whole wheat grains normally first produce the high-pI group after their germination (Sargeant, 1980), although a subset of the low pI gene family is expressed in aleurone of germinating grains (Huttly *et al.*, 1988). The same subset of low pI isozymes is also expressed in the maternal pericarp of developing grains, suggesting multiple regulatory elements for tissue specificity and GA responsiveness (Huttly *et al.*, 1988). It is hypothesised on the basis of conserved features in the O2S sequence in low-pI promoters, that this element is responsible for tissue specific expression of the promoter in aleurone or endosperm cells (Lanahan *et al.*, 1992). When the Box 5 and O2S elements (elements with apparent similar function) are transferred to an appropriate position in a high-pI promoter, the maximum level of transcription is raised 10-fold (Rogers *et al.*, 1994). Lenton and Appleford (1993) compared mRNA accumulation in scutellum and aleurone in near-isogenic lines (NILs) of the wheat variety Maris Huntsman, homozygous for either *rht3* or *Rht3* alleles. According to them results suggest that the Rht3 dwarfing gene prevents α -amylase mRNA accumulation more effectively in aleurone than in the scutellum.

2.3.3 Changes in hormone and enzyme activity during embryo and aleurone development

The accumulation of GA activity in developing grains of wheat occurs as fresh weight increases and peak levels are attained prior to maximum dry weight. Most of the GA present during development is in the endosperm, including the aleurone, testa and inner pericarp (Jacobsen and Chandler, 1988). As maturation proceeds, GA levels fall until in the near-mature grain the embryo contains the greatest amount of GA, although the total GA content of the seed at this stage is only a few percent of peak levels. Under normal conditions there is very little GA present in the dry wheat seed (Jacobsen and Chandler, 1988). For the first 48 hours of germination the scutellum is the major site of GA production and it is likely that the hormone is newly synthesised. It is also suggested that it is GA from the embryo which stimulates production of α -amylase in the aleurone, as removal of the embryo earlier than 24 hours after germination prevents an increase of GA in the endosperm and very little subsequent production of α -amylase. However, it has also been proposed that in barley, aleurone α -amylase production occurs in response to GA₄ synthesised in the aleurone layer. Aleurone GA₄ production would be in response to some unidentified component in the embryo (Jacobsen and Chandler, 1988).

Cereal grains accumulate ABA during development with maximum levels being reached generally after peak GA levels and just before the beginning of dehydration, after which ABA levels fall rapidly as the grain dries out. There is evidence that the fall in ABA levels occurs through enhanced metabolism of ABA. Per total grain weight peak ABA levels are at least an order of magnitude higher than maximum GA levels (Jacobsen and Chandler, 1988). Garcia-Maya *et al.* (1990) found in their study with the wheat variety Sappo, that ABA content of embryos peaked at 30 days post-anthesis and thereafter declined. The

hormone was however absent in isolated 30 days post-anthesis embryos. The increase in grain ABA levels during development could arise from novel synthesis of ABA within the grain, or from transport of ABA synthesised elsewhere in the plant into the grain via phloem, or from a combination of these processes. According to Jacobsen and Chandler (1988) studies have produced evidence to support the operation of both sources.

In isolated immature embryos low-pI isozymes are produced first, followed by the production of high-pI isozymes, though neither all the low- nor all the high-pI isozymes appear simultaneously (Garcia-Maya *et al.*, 1990). *In vitro*, most of the enzyme remains within the embryo, presumably the scutellum, and at 48 hours after embryo isolation only about 30% is secreted. α -Amylase production in isolated embryos is suppressed by ABA, although production of low-pI isozymes is less affected compared to that of high-pI isozymes (Garcia-Maya *et al.*, 1990). In (whole) wheat grain Mrva and Mares (1999a) found that 18 days post anthesis α -amylase activity is almost exclusively due to low pI enzyme. This enzyme disappears during normal grain development and ripening and only a residual activity remains at harvest ripeness. Garcia-Maya *et al.* (1990) found that very young (15 –20 days post-anthesis) embryos (of the wheat variety Sappo) are only able to produce the low-pI isozymes and a full complement of high-pI isozymes is only produced by 30 days post-anthesis material. They also found that the embryos' capacity for enzyme production declines in older embryos (after 35 days post-anthesis) and at later stages only the low-pI group is found. This can be overcome in isolated embryos of any age, by application of GA₃.

Aleurone tissue of the developing cereal grain does not normally produce α -amylase (Armstrong *et al.*, 1982) because the α -amylase genes are unable to respond to gibberellin (Cornford *et al.*, 1986), while aleurone tissue of mature grains produces copious amounts

of enzyme under the influence of the hormone (Jacobsen and Chandler, 1988). Similarly, embryos of developing wheat grains *in situ* do not contain α -amylase, though they do contain low-pI α -amylase transcripts, but these are not translated (Garcia-Maya *et al.*, 1990). A temporal change in GA responsiveness during development has been reported for wheat aleurone tissue (Gale *et al.*, 1983).

Working with the wheat variety Chinese Spring Mrva and Mares (1999a) found both low- and high-pI isozymes to increase with time in GA₃-treated de-embryonated mature grains (aleurone tissue), but the proportion of high-pI isozymes decreased from almost 70% to between 37 and 47%. After germination, low-pI isozymes only appear in the later stages. Their experiments also revealed that compared to germinating intact grains, GA₃-treated de-embryonated seeds synthesise relatively more low-pI isozymes and these appear much earlier. According to them the reason for this is not clear, but is presumably related to the level of GA₃ used (high). This would tend to mask any differences in dose response of the α -Amy1 and α -Amy2 system.

2.4 Pre-maturity α -amylase activity (PMAA)

Although pre-maturity α -amylase activity (PMAA) in the absence of sprouting is particularly marked in grain of certain wheat varieties, it is poorly understood (Kettlewell and Cashman, 1997). Traditionally empirical methods of selection against PMAA have been hampered by the sporadic nature of the problem (Gale and Lenton, 1987). Although it was quickly recognised that genetic susceptibility to PMAA expression is exposed by conditions that delay ripening (Flintham and Gale, 1988), subsequent studies have more clearly defined the environmental stimulus for PMAA (Major, 1999).

2.4.1 Environment and PMAA

Pre-maturity amylase is enhanced by many conditions, such as cool temperatures, precipitation, or high humidity such as occurs in a lodged crop, that extend the period of grain drying from maximum fresh weight to moisture levels too low for α -amylase formation (Gale *et al.*, 1983). Gold (1991) used polyethylene glycol (PEG) to manipulate the grain drying rate in detached cultured ears of Maris Huntsman NILs, but did not identify any relationship between grain drying rate and α -amylase activity. It can be argued though, that the relationship between α -amylase activity and grain drying rate becomes apparent at slower drying rates. Gold (1991) suggested that high humidity, high temperature, or a large diurnal temperature range might be responsible for stimulating high PMAA. In the variety Fenman higher levels of PMAA have been found to develop under conditions where both temperature and humidity increased relative to controls (Gold and Duffus, 1996). In contrast, an experiment with the wheat line BD-159 revealed that cool temperatures 21 and 28 days after anthesis increased the mean α -amylase activity in the grain (Mrva, 1994). BD-159 plants were transferred to a cooler temperature regime (12-17°C) for several days during the flowering period and then returned to a warm, dry environment to mature. The phenomenon was also noted under field conditions in years with low mean temperatures (approximately 15°C, compared to more normal temperature means of 18-20°C) during grain ripening. It is however unclear whether the effect of the low temperature is modulating PMAA by effects on grain drying rate or whether low temperature is actually stimulating expression of PMAA (Major, 1999). In UK commercial crops of 1985 PMAA was found to be high (Hough, 1990). In this year, temperatures during the first stages of grain growth were above average. In general, α -amylase activity rises rapidly in PMAA-susceptible varieties such as Fenman and is followed by a levelling off or a decrease in activity (Gold and Duffus, 1996). This levelling off or decreased

activity may be due to reducing moisture levels. Thus, according to Gold and Duffus (1996), the risk period may cover a rather narrow range of grain moisture content and may therefore explain why grain drying rates over a wider range are not always related to α -amylase activity. Experiments in controlled environment cabinets with a number of varieties has shown that the expression of PMAA may be dependent on a short, high temperature stress in early grain development followed by slow grain drying conditions (Lunn *et al.*, 2001a).

2.4.2 Morphology and PMAA

Evers *et al.* (1995) noticed that larger grained varieties consistently have low HFNs, to the extent that in an arbitrary category of the four lowest HFNs of each harvest within a certain period, the larger-grained types appeared disproportionately more frequent. Grain size has also been linked to the number of grains per ear (Abbate *et al.*, 1997), with grain size increasing as the number of grains per ear comes down.

Many large grains have a large endosperm cavity and Evers (2000) reported that the low HFN Australian variety Spica, produces large grains with a large endosperm cavity. Large endosperm cavities often apparently disappear towards maturity, as in a number of grains cavities tend to collapse (Greenwell *et al.*, 2001) (Plate 2.1 C). Results from experiments by Evers (2000) indicated that even in high HFN samples some enzyme activity occurs but the amount and the sites of origin differed for large and small grains. The source of enzyme in ungerminated Spica grains lay mainly in the aleurone of the crease region, while in another Australian variety Chinese Spring the enzyme was either absent or present mainly in the aleurone cells around the periphery of the grain.

Every *et al.* (2002) measured α -amylase activity in milled grain fractions of the Australian wheat variety Frame. They found the activity to be greatest in the germ and outer grain fractions. This suggests that a large proportion of α -amylase activity is derived from flour contaminated with outer grain parts and embryo/scutellum. With regards to PMAA as a source of amylase in the flour, these findings are consistent with observations by Cornford *et al.* (1987b) and Evers *et al.* (1995), that PMAA is greatest in the crease region of grains. Although the aleurone is situated on the outside of the starchy endosperm, the crease tends to be positioned towards the centre of the grain and thus according to Greenwell *et al.* (2001) highly likely to be incorporated into early flour streams during roller milling of the grain. Pre-maturity α -amylase would therefore play a role in the quality of even the highest grades of white flour.

2.4.3 PMAA physiology

The tendency to pre-maturity germination appears to be independent of a disposition to produce pre-maturity α -amylase (Gale *et al.*, 1983; Flintham and Gale, 1988; Flintham, 1990). The stage of onset of PMAA appears to be between 40% and 30% grain moisture (Gold, 1991) and enzyme production is halted at about 20% grain moisture (Gale *et al.*, 1983), with mature grain having a moisture content at harvest ripeness of <12% (Cornford *et al.*, 1987a). In wheat grains affected by PMAA, α -amylase isozymes controlled by *α -AMY-A1*, *B1* and *D1*, are synthesised in the aleurone tissue (Mrva and Mares, 1999a).

Pre-maturity α -amylase production is initiated and accumulated more rapidly at the embryo end of the grain (Gale and Lenton, 1987). Spatial differences in hydrolytic enzyme activity have been reported by Evers *et al.* (1995), within the aleurone area in wheat. Ritchie *et al.* (1999) found that in barley, aleurone cells closest to the embryo are more easily activated

at GA concentrations thought to occur naturally in germinating grain. Evers *et al.* (1995) found indications for higher α -amylase activity in the crease region of wheat grain. This accords to a considerable extent with findings reported by Cornford *et al.* (1987b), that pre-maturity α -amylase activity is greatest in the crease region of grains. A possible mechanism that fits these observations could involve the production, during the cell division phase of grain development, of an excessive number of aleurone cells in the cavity of grains, with these cells producing hydrolytic enzymes without the stimulation by growth substances, required by aleurone cells elsewhere in the endosperm (Evers *et al.*, 1995).

Other studies have reached similar conclusions. GA₁, thought to be one of the more potent α -amylase production inducing gibberellins, is only present in trace amounts at the time that pre-maturity α -amylase is formed in developing grain. Gale and Lenton (1987) found that in certain genotypes there is actually a decline in both concentration and amount of the most likely bioactive GAs and concluded that there was no direct evidence for a relationship between endogenous GA levels and pre-maturity α -amylase. They measured GA₁ and GA₅₄ levels in embryo and endosperm portions of developing grains at three different time points after anthesis. In both embryo and endosperm GA levels dropped during development. Gale *et al.* (1983) reported a premature window of aleurone responsiveness to GA in distal half wheat grains, which supposedly coincided with a period of limited detached whole grain germinability (embryo response). Gold and Duffus (1996) speculate that if PMAA relates to GA-sensitivity rather than to the amount of GA, some environmental trigger might switch on early GA sensitivity, thus extending the risk period for PMAA formation and with the moisture content at that stage determining whether or not α -amylase is synthesised. They further reason that in less susceptible varieties the onset of aleurone responsiveness may occur at moisture levels too low for α -amylase to be synthesised. In addition, Gold and Duffus (1996) speculate that an observed increase in the proportion of affected grains may be due to an increase in GA-

sensitivity of the aleurone layer. Grains vary considerably in their stage of development both within ears and between ears. That PMAA is not observed in all grains *in vivo* suggests that the response depends on a unique combination of physiological circumstances (Gold and Duffus, 1996). According to Norman *et al.* (1982), in addition to the requirement for exposure to an elevated temperature for a period of several hours, isolated aleurone tissue must also subsequently be subjected to a period at a lower temperature for just a few seconds in order to be made sensitive to GA. They placed containers with GA₃ treated grains (23 days post anthesis) in ice for 14 seconds. This decrease the temperature from 30°C to 25°C. They found that exposure of the aleurone to temperatures which induce sensitivity to gibberellic acid also results in an increased leakage of amino acids. It is suggested that the increase in sensitivity to gibberellin requires two separate processes to take place. One could be a homeoviscous adaptation of the cell membranes in response to elevated temperature, the other a subsequent permanent change in conformation of membrane components (Norman *et al.*, 1982).

2.4.4 The genetics of PMAA

2.4.4.1 Reduced height (Rht) genes influence PMAA expression

Currently, almost all of the commercial wheat varieties employ one of the *Rht* mutant alleles (Sun, 2000). Because common dwarfing (GA-insensitivity) genes reduce the expression of PMAA, according to Mrva and Mares (1996b), it is likely that gibberellin is involved in the response either directly, via increases in GA concentration or sensitivity, or indirectly, via a reduction in mechanisms which prevent grains from responding to GA prematurely. In fact, GA-insensitive dwarfing genes have effects on both pre-maturity

α -amylase and post-dormancy α -amylase accumulation (Gale and Lenton, 1987). In a tall Maris Huntsman line lacking GA-insensitive dwarfing alleles, pre-maturity α -amylase production is initiated and accumulated more rapidly at the embryo end of the grain, with both α -AMY1 and α -AMY2 isozymes present. Gale and Lenton (1987) reason that although this could reflect scutellum produced α -amylase diffusing into the endosperm it may also represent aleurone produced α -amylase initiated in response to GA produced in the embryo. They observed that in tall wheat lines, pre-maturity α -amylase appears to accumulate without a corresponding increase in endogenous GA and concluded that the fact that the Rht genes reduce the production of premature α -amylase, does not appear to be related directly to the ability to make tissue insensitive to applied GA. However, Lenton and Appleford (1993) found that a considerable proportion of α -amylase production in germinating *rht3* wheat grain is GA-dependent, although it may be less so in grain of shorter Rht3 lines.

Lenton and Appleford (1993) also found that lowering the germination temperature from 25°C to 15°C completely overcame the inhibitory effect of the Rht3 allele on α -amylase production, during germination in Maris Huntsman NILs. They concluded that Rht3 prevents α -amylase mRNA accumulation more effectively at higher than lower temperatures, is more active in aleurone than scutellum and against the low-pI than the high-pI gene family.

The two Rht loci of wheat, Rht1 (recently re-designated Rht-B1b) and Rht2 (recently re-designated Rht-D1b), are orthologs of GAI, a GA-derepressible repressor of GA-mediated growth responses in *Arabidopsis* (Sun, 2000), though to date there is no direct evidence for a role for GAI in GA signalling in aleurone cells (Lovegrove and Hooley, 2000).

Presumably, a deletion in the RHT protein (produced by a *Rht* allele) locks the protein into

a conformation that can no longer, or only very weakly, respond to the GA signal (Peng *et al.*, 1999). The Rht genes probably upregulate bioactive GA production via an unknown feedback mechanism (Sun, 2000).

2.4.4.2 PMAA gene(s)

An examination of F₄-lines from the cross Professeur Marchal (thought to be the source of PMAA in UK breeding programs) x Nord Desprez, established control of high α -amylase by either a single, or at most two duplicate recessive genes (Bingham and Whitmore, 1966). This finding is supported by other studies examining distribution and segregation patterns, indicating a consistency with control at a single locus and with high α -amylase the recessive allele (Mares and Gale, 1990; Mrva and Mares, 1996a; Mrva and Mares, 1999a). This putative recessive gene, however, controls pre-mature α -amylase through the appearance of high pI isozymes controlled by genes on all three group 6 chromosomes, similar to the pattern observed in the early stages of germination (Mares and Gale, 1990). Although the genes controlling pre-maturity α -amylase are independent of those controlling dormancy, development of high levels of enzyme before dormancy may be associated with more rapid sprouting, following dormancy break (Gale and Lenton, 1983). Flintham and Gale (1998) put forward that eliminating the PMAA-gene(s) from breeding programmes should be straight forward, provided it is appreciated that the recessive nature will prevent detection for at least one generation, that expression is dependent on ripening conditions and that expression can be confounded by the presence of grains in which germination has been initiated, when α -amylase is measured in harvested grain. Mrva and Mares (1999b) speculate that the PMAA gene is related to, or interacts with, the putative gene(s) involved in the GA-induced synthesis of α -amylase, which in turn may be part of a complex sequence that includes the *GAmyb* gene, reported by Gubler *et al.* (1995).

2.4.5 PMAA in present-day UK winter wheat varieties

Although initially thought to be a relatively minor problem (Flintham and Gale, 1988), PMAA has eluded selection by breeders against it, and is still present in currently cultivated UK winter wheat varieties. Research into PMAA has up until now been mainly done on older varieties such as Maris Huntsman and Fenman (Gale *et al.*, 1983; Gale and Lenton, 1987; Flintham and Gale, 1988; Gold and Duffus, 1996). However, more recent UK winter wheat varieties have evolved towards higher yielding and shorter phenotypes, although many more traits have also drifted away from the old standards, either intentionally or unintentionally. Other differences with the older varieties are as varied as shifts in vernalisation requirement to differences in grain protein content and composition. Some of the changes are as a result of modern accelerated breeding techniques and molecular and biochemical marker selection, not used in the development of older varieties.

When in 1999 Rialto crops produced low HFN with poor breadmaking quality, some farmers incurred financial loss due to waning anticipated breadmaking premiums. Breeders at Monsanto (UK) could not attribute the shortfall in quality solely to sprouting. It was hence proposed that some of the reduction in breadmaking quality could be related to PMAA in the grain (section 2.1 describes how pre-maturity α -amylase affects breadmaking quality).

Rialto is still actively used in current breeding programs and is in the parentage of some of the latest varieties on the market. This project was established to assess PMAA in a modern UK winter wheat genetic background. Markedly, a great deal of focus was trained

at the variety Rialto. However, work on field and controlled environment (CE) grown material would give a greater insight into the induction and accumulation of PMAA in a range of wheat genotypes (chapters 4 and 5). The ultimate aim of this project was to lay down the foundation for the development of a breeders' screen for PMAA (largely addressed in chapter 6).

3 General materials and methods

Lunn *et al.* (1996) demonstrated that it is possible to discriminate between different types of grain α -amylase activity using a combination of methods (Table 3.1). This combination of methods was used in the analysis of grains from field grown cv. Rialto (chapter 4), CE grown plants (chapter 5) and detached tillers (chapter 6).

Sprouting was scored both visually and using the more objective fluorescein dibutyrate (FDB) method (Jensen *et al.*, 1984). The FDB method visualises sprouting related secretion of the hydrolysing enzymes esterase and lipase by the embryo/scutellum into the endosperm. Iso-electric focussing was used to identify the α -amylase isozymes produced.

Table 3.1 Tests used to discriminate between various sources of α -amylase activity in wheat grain

Test	RPAA	PMAA	PrMS	PoMS
Visual sprouting	No	No	Yes	Yes
FDB	-	-	+	+
IEF	AMY2	AMY1	AMY1/2	AMY1/2

Lunn *et al.* (1996)

As PMAA present in the grain is expressed mostly in the crease (Cornford *et al.*, 1987b) (also see section 2.4.2) the location of α -amylase secretion in the grain was visualised on Phadebas gel (Major, 1999; Greenwell *et al.*, 2001). A colorimetric microtitre plate assay was used to measure the α -amylase activity of milled grain samples.

3.1 Principles of analytical methods

The iso-electric focussing (IEF) technique utilises the principle of ion migration over a pH gradient, established by the application of an electric field (Chang, 1981). The pH gradient

is established between electrodes positioned at opposite ends of a polyacrylamide gel. After a protein mixture is placed near one of the electrodes different proteins will migrate to form stationary bands along the gradient at points where the pH is equal to their isoelectric (pI) points. Following separation, the protein bands of interest (α -amylase isozymes) are made visible by first washing the gel in a starch solution followed by iodine staining. AMY1 does not migrate as far as AMY2. As a result, the two isozyme families can be visualised separately as two distinct clusters, when present (Plate 3.1). PMAA consists mainly of AMY1 (Gale *et al.*, 1983; Major *et al.*, 1996).

The Phadebas gel method, a relatively simple technique for *in situ* localisation and identification of grain α -amylase activity, was developed at the Campden and Chorleywood Food Research Association (CCFRA) (Greenwell *et al.*, 2001). This method is similar to the older beta-limit dextrin blotting assay, but is simpler as it does not require staining. It was developed from the diffusion-well procedure (Hejgaard and Gibbons, 1979) using Phadebas blue starch as a substrate specific for α -amylase. This special starch is produced by cross-linking partially hydrolysed potato starch with 1,4-butanedioldiglycidether (Barnes and Blakeney, 1974). The number of cross-linking bridges regulates swelling of this three-dimensional insoluble lattice network. It also controls susceptibility to enzyme attack. The blue colour comes from Cibachron Blue, attached to the substrate by covalent bonds. Blue dye released from the insoluble lattice network during digestion of the gel comes away with the grain sections at the end of the blotting process or is carefully washed away with deionised water.

Total α -amylase activity in milled wheat samples was measured with a colorimetric microtitre plate assay, using a commercially available kit based on a method developed by McCleary and Sheehan (1987) for specific determination of α -amylase. The air segmented

flow analyser used earlier by Major (1999) at Harper Adams proved to be difficult to operate and would only allow the analysis of one sample at a time. In contrast, the microtitre colorimetric assay, adapted from an assay reported by Sirou *et al.* (1990), offered the ability to quantify α -amylase activity for a large number of samples in a relatively short period of time. The assay is capable of measuring the α -amylase activity in whole-wheat grains, half grains, dissected fractions of wheat, or flour, weighing 0.01g and above (Greenwell *et al.*, 2001) and incorporates the Randox test kit (Randox Laboratories, AY891) for determining α -amylase activity in blood serum or urine. The method uses benzylidene blocked p-nitrophenyl-maltoheptaoside as substrate for α -amylase action. Two indicator enzymes, glucoamylase (to cleave the amylase reaction products) and α -glucosidase (to release the p-nitrophenol), are incorporated in the substrate mixture.

Plate 3.1 α -Amylase IEF. At similar extraction volume; *left* severe sprouting, smearing of AMY1 bands (below) and distinct AMY2 bands (top), *right* possible PMAA, distinct AMY1 bands (below) and no or faint AMY2 bands (top)



Band positions were determined relative to Barley α -amylase (Sigma A-2771), with 72 h sprouted samples produced from clean unsprouted grain. Barley type B amylase at pI 5.9-6.6 and type A amylase at pI 4.6-5.2 (Rogers, 1985)

The substrate is chemically blocked to prevent cleavage by the indicator enzymes prior to digestion by α -amylase. α -Amylase extracts are incubated with the substrate solution under defined conditions in 96 well flat-bottom plates, thus making it possible to analyse multiple samples simultaneously. With test conditions standardised the amount of p-nitrophenol released depends on the α -amylase activity of the extract solution and is measured spectrophotometrically.

3.2 Detailed methods used in grain analysis

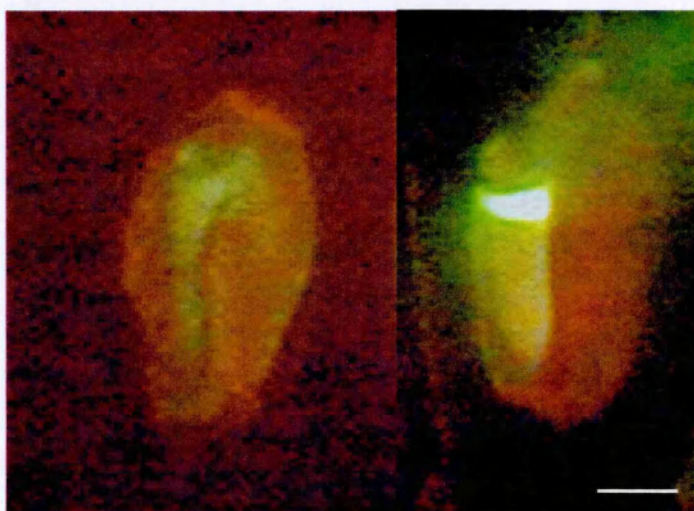
A visual inspection for sprouting was carried out prior to further analysis of the grain. Grains were scored as sprouted when they exhibited a crack in the pericarp covering the embryo or clear radicle emergence (Wellington, 1956).

3.2.1 Fluorescein dibutyrate test

The Fluorescein dibutyrate (FDB) test was used to identify lipase/esterase activity indicative of germinative processes (Jensen *et al.*, 1984) in longitudinal sections of grain. A Danbrew Malt Modification Analyser Kit (Danbrew, Denmark) was used for the analysis and included a seed fixation system with press and holder, a disc sander, self-adhesive sanding paper, seed matrix and clay blocks. Grains were placed on the matrix with the crease showing sideways and were pressed into a cernit clay block. The blocks were placed on the disc sander and sanded down until a longitudinal section through the crease was showing.

Using a magnetic stirrer 236 mg FDB (product number 46942, Sigma) was dissolved in 80 ml 99% ethanol. The volume was made up to 100 ml with distilled or deionised water. Clay blocks with embedded half grains were stained with the FDB solution for 10 min and incubated in distilled or demineralised water for 2 min at 60°C. Excess water was carefully removed with paper towel. Blocks were placed in the analyser, under a UV light source. Lipase/esterase activity was seen as a yellow fluorescence in the grain (Plate 3.2).

Plate 3.2 FDB stained longitudinal grain sections with embryo at top end; Sound grain left, Sprouted grain right*



* yellow fluorescent area expands further into endosperm as severity of sprouting increases. Bar corresponds to 1 mm.

A maximum of 50 grains per block were stained with FDB and the number of grains showing fluorescence in the embryo/scutellar region recorded.

3.2.2 α -Amylase iso-electric focussing

A commercially available electrophoresis kit (Pharmacia LKB, Sweden) was used for iso-electric focusing (IEF) of wheat α -amylase isozymes. The kit consisted of a Multitemp II thermostatic circulator for cooling gels during IEF runs, a programmable Multidrive XL powerpack and a Multiphor II gel tank containing a gel plate with cooling capability.

Amylase was extracted from flour samples by weighing 0.15 to 0.40 g of flour into 1.5 ml centrifuge tubes and adding 1 ml extraction solution. The extraction solution consisted of 0.34 M sodium chloride (20 g/l NaCl) and 1.26×10^{-3} M calcium acetate (0.2 g/l $\text{Ca}(\text{C}_2\text{H}_3\text{O}_2)_2$). The samples were whirl mixed for three seconds. Suspended samples were incubated for 1 h in a 30°C water bath and whirl mixed every 15 min to re-suspend precipitated flour. After this procedure the centrifuge tubes were placed in a micro-centrifuge and spun for 10 min at a relative centrifugal force (rcf) of 15626 g (13000 r.p.m. and 82.7 mm radius). Supernatant was pipetted into a fresh 1.5 ml centrifuge tube and incubated at 70°C for approximately 15 min in a lidded water bath in order to inactivate β -amylase. The heat-treated samples were re-spun for 10 min at 15626 g and checked against a non-treated sprouted sample.

Polyacrylamide gels (80-1124-80, Ampholine PAGplate pH 3.5-9.5, Amersham Pharmacia Biotech AB) were placed on the gel plate cooled to 10°C in the Multiphor II gel tank. The gels were insulated from the gel plate with 1 ml kerosene (60710, Fluka). Electrode strips were cut so that they were of a slightly shorter length than the gels.

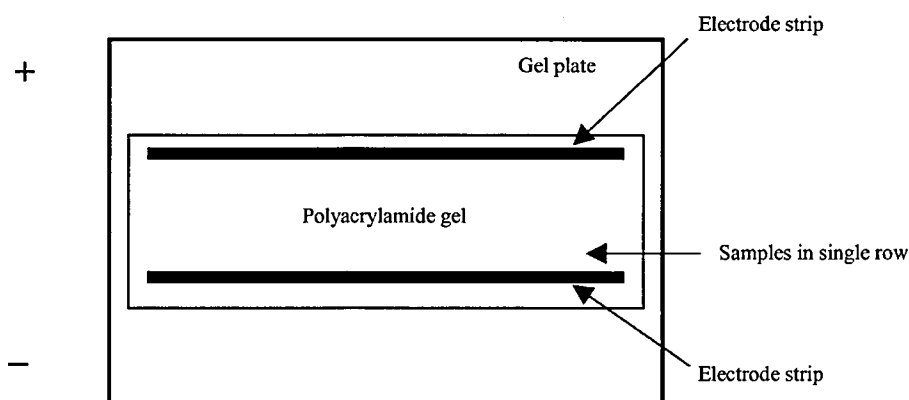


Figure 3.1 Diagram of a gel plate. Samples are arranged along the cathode in a single row

The electrode strips were soaked in 3 ml of electrode buffer (cathode 0.1 M NaOH and anode 0.1 M H₃PO₄) and placed on the gel surface (Figure 3.1). Applicator strips were placed in a single row at a fixed distance from the cathode. Between 15 and 30 µl of supernatant was pipetted onto the applicator strips, one aliquot per sample position. Electrode wires were lowered onto the electrode strips and an electric field applied across the gel. Gels were run at 1500 V and 50 mA for 1.5 h.

After removing electrode and applicator strips gels were washed in 1% (10 g/l) soluble starch (85645, Starch from potatoes for electrophoresis, Fluka Biochemika) for 10 to 30 min at room temperature. Excess starch was removed by washing the gels three times with distilled or demineralised water. Isozyme bands were made visible with a 4% I₂/KI solution (4% solution made up with 10 ml stock and 250 ml distilled or demineralised water, with stock solution 24.8×10⁻³ M or 6.5 g/l I₂ and 117.5×10⁻³ M or 19.5 g/l KI). When a brown-purple staining of the gel became apparent the iodine solution was poured away and the gels immediately placed on a light box situated below a pivot-mounted camera. Pictures were taken on standard photographic film and negatives were scanned as uncompressed high quality TIF image files.

3.2.3 Visualising the location of grain α-amylase activity

Agarose/Phadebas gel was made by combining a heated agarose solution with a Phadebas paste. The agarose solution was prepared by dissolving 0.132 g low melting point agarose (Agarose A5030, Type IX, Ultra-low Gelling Temperature, Sigma) in 10 ml distilled water on a magnetic stirrer at 50°C. The Phadebas paste was made by adding 0.264 g Phadebas powder (Pharmacia Special Blue Starch, Pharmacia Sweden) to 10 ml warm distilled water in a 40 ml beaker suspended in an ultrasonic shaker filled with 30-50°C water. Disposable

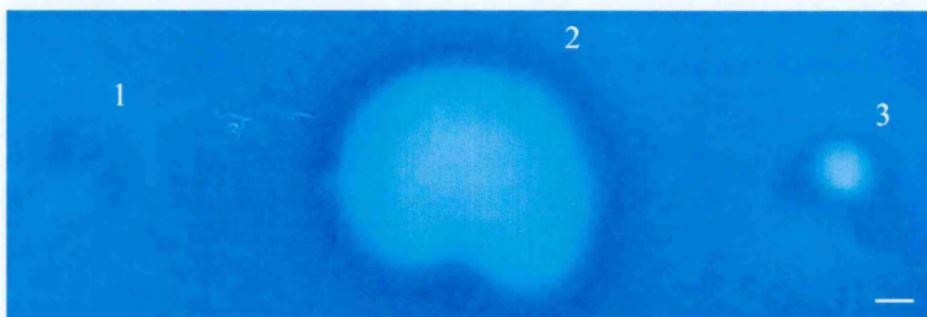
Petri dishes and pipette tips were kept warm in a 55-60°C oven. The agarose solution was added to the Phadebas paste and immediately transferred to a magnetic stirrer where the two components were mixed vigorously while maintained at 50°C. Alternatively, Phadebas powder was slowly added to a 20 ml agarose solution, in small amounts, after the agarose was fully dissolved, making sure that all Phadebas powder was incorporated into the mixture before subsequent amounts were added. The Phadebas gel was mixed vigorously for several additional minutes on a magnetic stirrer at 50°C.

Warm Petri dishes were placed on a level hot plate at 30-40°C and a layer of gel, approximately 1 mm thick, was pipetted into the dishes. The volume (V in ml) of Phadebas gel to be added to a Petri dish was calculated using the following formula $10^3 V = \pi \times (\frac{1}{2} D)^2$, with D the diameter of the Petri dish (D in mm). Gels were left to set on a level surface for 5 min. The gels were fully solidified on ceramic tiles cooled to -20°C. Gels were sealed with cling film and kept away from direct sunlight or other heat sources, or stored in a fridge (5-8°C) when kept for longer periods (maximum of seven days). All Petri dishes used had a diameter of less than 90 mm, as the larger the Petri dishes used the more difficult it became to cover the base of a dish evenly.

Using a single edged razor blade or scalpel grains were sliced either transversely (perpendicular to the length of the seed) behind the embryo, approximately half of a grain length from the embryo proximal end, or longitudinally through the crease. Cut surfaces were brushed with deionised or distilled water and placed on the gel surface. Petri dishes were sealed with cling film and incubated at 40°C. Grains were carefully removed, after gels were taken out of the incubator and re-solidified on cold ceramic tiles. Digested gel was removed with the grain or was carefully washed away with deionised or distilled water. Size and position of the clear patches formed on the Phadebas gel as a result of

α -amylase activity gave an indication as to the source of the amylase (Plate 3.3). Sprouting could be detected within an hour. PMAA detection took between 3 and 4 h. If necessary, gels were viewed over a light box.

Plate 3.3 Footprints of transversely sliced grain sections incubated on Phadebas gel;
1 Sound grain, 2 Sprouting, 3 PMAA



Bar corresponds to 1 mm

3.2.4 α -Amylase activity

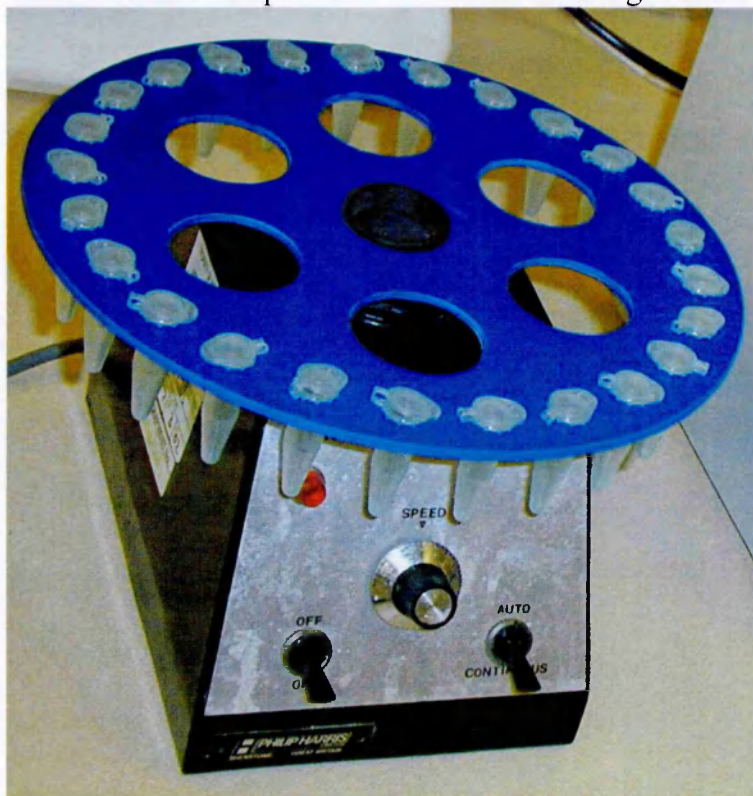
Flour was weighed into 1.5 ml centrifuge tubes and extraction buffer (Table 3.2) added, with the volume of extraction buffer in μ l being 10 times the weight of the sample in mg. Each sample was whirl mixed for two seconds and shaken for 30 min in a multi-sample whirl mixer (modification of a construction used by Bhandari, personal communication) (Plate 3.4). Shaken flour/buffer mixtures were spun in a micro-centrifuge at an rcf of 15626 g (13000 r.p.m. and 82.7 mm radius) for 10 min.

Table 3.2 Reagents used for determining sample α -amylase activity

Solution	pH adjusted to	Quantity	Components	Quantity of Component
Extraction buffer	5.2	250 ml	malic acid	50×10^{-3} M (1.675 g)
			sodium chloride	50×10^{-3} M (0.73 g)
			calcium chloride	1.5×10^{-3} M (0.055 g)
Amylase substrate solution	-	approx. 5 ml	reconstitution buffer	5 ml
			amylase substrate	1 vial
Reconstitution buffer	5.0	50 ml	malic acid	150×10^{-3} M (1.006 g)
Stopping reagent	-	25 ml	Tris(hydroxymethyl)-methylamine	82.5×10^{-3} M (0.25 g)

Buffers were pH adjusted with concentrated (2-10 M) NaOH. All solutions were made up to final volume with distilled water.

Plate 3.4 Multi-sample mixer for 1.5 ml centrifuge tubes



Modification of a construction used by Bhandari (personal communication)

After centrifuging, 30 μ l of supernatant was pipetted into a flat-bottomed 96 well microtitre plate well (ELISA plate, Greiner bio-one Ltd.). Substrate (amylase test kit AY891, Randox Laboratories Ltd.) solution (Table 3.2) was added, 30 μ l per sample, and plates incubated for 20 min at 32°C. A saturating amount (150 μ l) of Tris (product code 80-1128-82, Pharmacia) solution was added to stop the reaction. The microtitre plates were gently tapped to remove air bubbles. Absorbance was read at 405 nm in a Bio-Rad Benchmark Microplate Reader (Bio-Rad Laboratories Ltd.). The absorbances were corrected against two time blanks. In time blanks, α -amylase degradation of the substrate solution was prevented by addition of the stopping reagent prior to that of the substrate solution. All samples were analysed in duplicate. The within-run precision of assays of two dilutions of two extracts is estimated to give a CV of approximately 2% (Sirou *et al.*, 1990). The linear range of a series of dilutions was determined using commercially available cereal α -amylase (A-2771, type VIII-A from barley malt, Sigma). The α -amylase was dissolved and further diluted in α -amylase extraction buffer (Table 3.2). If necessary,

extracts from milled samples were diluted in order to fit absorbances within the linear range. Absorbances were converted into enzyme units using the following formula:

$$c = \frac{10^3 A_{405} \pi D^2}{4 \epsilon t w} \text{ in mEU/mg} \quad \text{adjusted from Sirou } et al. (1990) \text{ (Greenwell } et al., 2001)$$

With ϵ the molecular extinction coefficient of p-nitrophenol, D the microtitre plate well diameter (cm), t the time of reaction (min) and w the weight of the sample in the reaction volume (mg). Mathewson and Seabourn (1983) took the ϵ for p-nitrophenol to be 18800 mol⁻¹l⁻¹cm⁻¹ assuming pH>10. One unit of activity was defined as the amount of enzyme required to release 1 µmol/min of p-nitrophenol under test conditions (Sirou *et al.*, 1990).

4 The occurrence of pre-maturity α -amylase activity and its relationship with the pre-harvest environment of field grown cv. Rialto

4.1 Introduction

The propensity of field-grown wheat to produce pre-maturity α -amylase activity (PMAA) under appropriate weather conditions has been described for different varieties. Gold (1991) reported differences in PMAA expression between winter wheat varieties. Major (1999) described variations in PMAA susceptibility for four French winter wheat varieties and four UK varieties chosen for their difference in Hagberg falling number (HFN) and sprouting resistance. Late maturity α -amylase (LMA) activity, a problem similar to PMAA, has been described extensively by Mrva and Mares (1996a, 1996b, 1996c, 1999a, 1999b and 2001a) for spring wheats in Australian.

An unexpectedly low HFN is still a sporadic occurrence in UK wheat crops, reducing the price farmers received for their grain, with drops in HFN more apparent in some years (Lunn *et al.*, 2001a and 2001b). Although not found during the variety selection program, the Monsanto (UK) Ltd. group 2 winter wheat variety Rialto has, after its release, produced these unexpectedly low HFNs.

As Rialto was not expected to have sporadic and unpredictable drops in grain quality, later suspected to be caused by PMAA, it is important to establish conclusively the presence of PMAA in the present UK commercial stock, as this will show if PMAA is a problem in current breeding programs (also see section 2.4.5). Rialto, a modern short and high

yielding winter wheat with a Haven × Fresco parentage (Anonymous, 1996), could have inherited a PMAA gene from cv. Professeur Marchal (Bingham and Whitmore, 1966).

If it is shown that PMAA persists in wheat breeding lines, breeding strategies will have to be adapted. Samples were gathered from Rialto crops in order to test the hypothesis that cv. Rialto is PMAA-susceptible. In addition, weather data collected for the different sites was examined in combination with α -amylase measurements. Identifying meteorological parameters that could potentially be linked to PMAA formation in the field would aid the development of a PMAA screen for plant breeders by helping to define a specific PMAA stimulus.

4.2 Materials and methods

4.2.1 Grain samples

Grain samples were taken from bulk harvested grain from 13 year-location combinations. Table 4.1 shows the sites where and the years in which the Rialto crops were grown. All year-location combinations were either commercial or yield trials. From the bulked grain of each combination a 25 g sample was taken (Simon, 2000) except for two samples designated A024 and D023 of which fewer grains were available.

Table 4.1 Origin of cv. Rialto grain samples. Crops were grown in three years and seven different locations in the UK

Year	Location	Designation
1995	Grantchester (Cambridgeshire)	A024
1998	Grantchester (Cambridgeshire)	D023
1998	Woodbridge (Suffolk)	D179
1998	Dereham (Norfolk)	D204
1998	Rutland (Leicestershire)	D234
1998	Dereham (Norfolk)	BJB
1999	Dereham (Norfolk)	AJB
1999	Trumpington (Cambridgeshire)	E15
1999	Trumpington (Cambridgeshire)	E16
1999	Fullerton (Hampshire)	E153
1999	Shipdham (Norfolk)	E194
1999	Rutland (Leicestershire)	E320
1999	Dereham (Norfolk)	E327

All grain samples were taken from bulk harvested grain and consisted of a minimum of 600 grains except for A024 and D023 of which fewer grains were available.

An electric coffee grinder was used to roughly process 100 grains and break down the pericarp. Except for samples A024 and D023 of which 50 grains were used. Samples were milled for at least 50 s. The rough flour was further processed with pestle and mortar for at least 3 min. Before processing subsequent samples in the coffee grinder, flour residues were removed by thoroughly wiping the grinder well with paper towel and air cleaning the well and blade mechanism with compressed air. Milled samples were stored at -20°C prior to analysis. As the results from various analyses done on the milled samples were to be compared with results in relevant publications, the store grain samples were processed as they were and not freeze-dried prior to milling. The mean and s.d. of the dry matter fraction of the stored grain samples was $0.965 \text{ g} \pm 0.002$.

A sprouted control was produced from an unsprouted grain sample by germinating the latter in a controlled environment (CE). The sprouted control was used as a reference for the Rialto samples during FDB and IEF analysis. Grain from a cv. Chaucer crop were used as it contained no visible signs of sprouting in the sub-sample taken and produced no FDB fluorescence in individual grains. Grains were placed crease down in a 90 mm Petri dish containing one layer of Whatman No.1 filter paper and 5 ml of distilled or demineralised

water was added. The number of grains per Petri dish was limited to a maximum of 40. Closed Petri dishes were placed in a CE cabinet (Fitotron, Sanyo Gallenkamp plc.) maintained at 20°C, with a 10 h light period (full fluorescent lighting) and 50% relative humidity. After 72 h the grains with roots of 5-10 mm were placed crease down on a dry piece of Whatman No.1 filter paper within the CE cabinet for at least another 24 h in order to dry. For analysis the grains sprouted in CE were processed in a similar way as the grains from the Rialto samples.

4.2.2 Weather data

Data on maximum and minimum daily temperature spanning the months June, July and August, thus covering the grain development period, was acquired from Met Office weather stations (British Atmospheric Data Centre, 2002) and from Monsanto (UK) Ltd. on-site weather data logbooks. Met Office stations Charsfield, Morley St Botolph, Cottesmore and Leckford were chosen for their proximity to the trial sites in Woodbridge, Dereham/Shipdham, Rutland and Fullerton respectively. Nearest weather data for the Grantchester and Trumpington sites was provided by Monsanto (UK) Ltd. on-site (Trumpington) meteorological measurements. For days when there was no data recorded, data points were entered for analysis by linear interpolation. Diurnal temperature difference (ΔT) was calculated by subtracting the measured daily minimum temperature (T_{\min}) from the measured daily maximum temperature (T_{\max}). A quasi-quantitative expression of grain development was calculated using the formula $\Sigma((T_{\max} + T_{\min})/2)$. Units are expressed in °Cdays.

As there were no detailed records available on crop husbandry for the 13 Rialto crops, the AFRC-model was used to estimate the onset of various grain development stages (Weir *et al.*, 1984).

4.2.3 Grain analysis

A visual inspection for sprouting was carried out on 100 grains of each sample and 50 grains per sample were stained with FDB. In addition, 72 h germinated grains of cv. Chaucer were FDB stained as controls, one for each 50 grain sample. IEF, Phadebas gel test and total sample α -amylase measurements were carried out as described in chapter 3.

4.2.4 Statistical analysis

An indication as to the equality of two variances was supplied by an F-test and testing for the difference of two means was done with a t-test (section 4.3.2). Coefficients of product-moment correlation, or simply correlation coefficients, were calculated and correlation matrices for various weather and α -amylase parameters generated in Microsoft Excel 97. Significant values ($P < 0.05$) in correlation matrices were identified using Table A18 in Campbell (1989). Linear regression analysis including multiple linear regression was carried out in Genstat for Windows 6th edition. All data considered not to be normally distributed was transformed to a different scale to better fit a normal distribution.

4.3 Results

4.3.1 Sprouting assessment

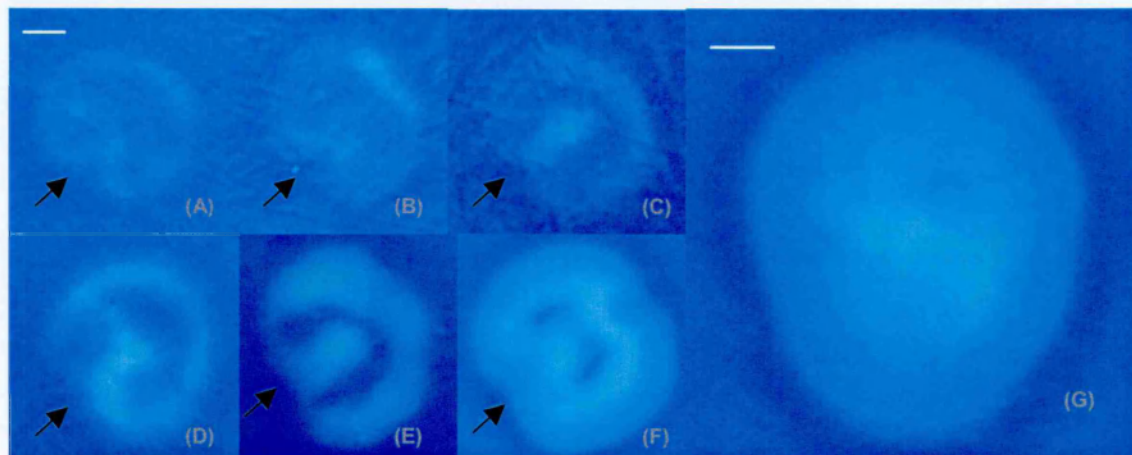
A visual inspection of the grain showed no apparent sprouting in the single sample from 1995, two sound samples were identified out of five collected in 1998 and only one non-sprouted sample out of seven from 1999. The percentage sprouted grains per sample ranged from 0% in all three years to 11% in 1999 (Table 4.2). Visible sprouting in 1998 and 1999 averaged 1% and 4% respectively.

Due to the size of the samples only 50 grains per sample were assessed with the destructive FDB technique. FDB analysis supported the finding of the visual inspection, that there was no sprouting in the 1995 sample. Esterase/lipase activity was only found in one of the five samples from 1998. In contrast, five of the seven samples collected in 1999 showed sprouting related enzyme activity (Table 4.2).

Sprouting was also observed on Phadebas gel. Both sprouting and PMAA-induced grains trigger α -amylase secretion from crease aleurone. It was therefore important to confirm that differences between the two sources of α -amylase, found on Phadebas gel for a range of varieties (Major, 1999; Greenwell *et al.*, 2001), were apparent for cv. Rialto. Over two hundred Rialto grains from a high HFN crop and selected for their unsprouted appearance were germinated in a controlled environment, as described in section 4.2.1, for 40 to 100 h. As the imbibition time increased so did the α -amylase degradation of Phadebas gel (Plate 4.1). In most grains imbibed for 50 to 70 h there was comparatively uniform low-intensity α -amylase activity noticeable in the aleurone (A). At around 70 h sprouting α -amylase activity increased noticeably, frequently observed to start as an ostensible widening of the

digested area in the dorsal aleurone region (B), followed regularly by a marked increase in activity in the crease region (C and D). The digested area increases in size as severity of sprouting increases (E and F), to a point where highly sprouted grains produce clear halos (G) on Phadebas gel, extending to several millimetres outside the grain outline (also see Plate 3.3).

Plate 4.1 Phadebas gel footprints of transverse sections of sprouted cv. Rialto grains



Arrows show position of crease. Picture A: sprouted between 40-50 h, 2.5 h incubation on Phadebas gel. Pictures B-F: between 70-90 h and G: 100 h sprouting, with 1 h incubation on Phadebas gel. Root and plumule development illustrated in Plate 5.10. Bars correspond to 1 mm.

4.3.2 α -Amylase analysis

All year-location combinations had grains that produced α -amylase related discoloration when incubated on Phadebas gel (Table 4.2). The amount varied between 26% to 78% of the total number of grains in a sample. Clear PMAA in grains was identified as enzyme activity only present in the crease region. Only in the 1995 sample was all α -amylase activity identified on Phadebas gel due to PMAA. Of the grains showing α -amylase activity in the other samples PMAA grain varied between 36% and 83%. Relatively few grains displayed a high amylase activity, identified by an area of discoloration larger than the grain outline, indicating the presence of only a small number of severely sprouted grains.

Table 4.2 Number of visibly sprouted and FDB fluorescent grains, sample enzyme activity and α -amylase visualised on Phadebas gel, for Rialto samples collected in three years

	Designation	Visible sprouting	FDB fluorescence	Amylase activity (mEU/g fw)	Phadebas gel		
					Number of grains showing α -AMY activity (%)	Percentage of grains showing α -AMY activity solely in the crease region (% in the population of grains with α -AMY activity)	Number of grains with high α -AMY activity* (% in the population of grains with α -AMY activity)
1995	A024	0	0	10	13 (26)	26 (100)	0 (0)
	D023	2	0	240	23 (46)	38 (83)	0 (0)
	D179	2	0	120	36 (72)	40 (56)	1 (3)
1998	D204	0	1	280	39 (78)	44 (56)	3 (8)
	D234	1	0	260	30 (60)	38 (63)	1 (3)
	BJB	0	0	450	26 (52)	32 (62)	1 (4)
1999	AJB	3	1	520	32 (64)	40 (63)	1 (3)
	E15	0	0	170	27 (54)	42 (78)	1 (4)
	E16	5	1	290	14 (28)	10 (36)	2 (14)
	E153	5	3	700	23 (46)	24 (52)	7 (30)
	E194	11	3	1030	29 (58)	40 (69)	5 (17)
	E320	1	0	190	26 (52)	40 (77)	1 (4)
	E327	4	2	290	23 (46)	32 (70)	6 (26)

Sample size for visible sprouting and FDB is 100 and 50 grains respectively. Phadebas analysis done on 50 grain samples.

* Area of activity at least 1 mm larger than grain outline.

α -Amylase activity of the sample taken in 1995 was negligible compared to the α -amylase activity of samples collected in 1998 and 1999. While the activity from the 1995 sample was 10 mEU/g fw, enzyme activity varied between 120 to 450 mEU/g in 1998 and 170 to 1030 mEU/g in 1999 (Table 4.2). Although the average α -amylase activity in 1999 was higher than the average for 1998 (Figure 4.1) it cannot be shown convincingly (level of significance 0.05) that 1999 samples produced systematically higher α -amylase activities than those from 1998. The same result was found for the natural logarithm of α -amylase activity.

Iso-electric focussing showed no significant AMY2 activity in most of the Rialto samples. While a second 50 h sprouted control of cv. Chaucer, bearing main roots of approximately 2 mm, produced clear and conspicuous AMY2 bands on polyacrylamide gel no substantial AMY2 activity was detected for any of the Rialto year-location combinations. Exceptions to this were the 1999 E194 Shipdham and E153 Fullerton samples, which did produce faint

AMY2 bands.

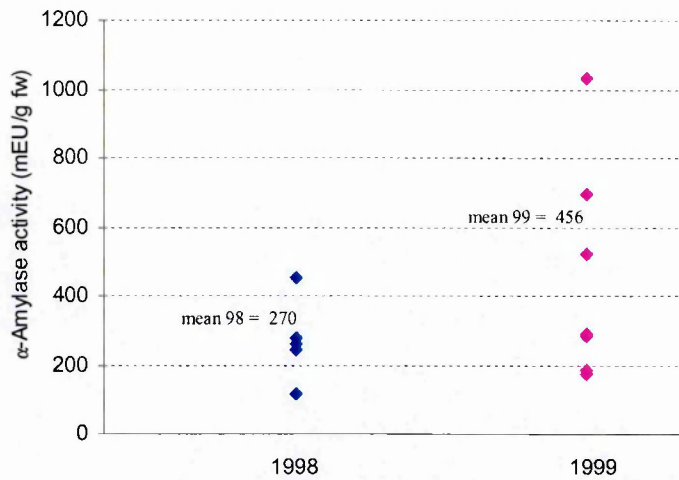


Figure 4.1 α -Amylase activities for samples collected in 1998 and 1999 and their respective means

4.3.3 α -Amylase activity and sprouting

Both the number of visibly sprouted grains and the number of grains showing esterase/lipase activity related FDB fluorescence are positively correlated with increasing α -amylase and have an adjusted R^2 * of 0.628 ($P < 0.001$) and 0.616 ($P < 0.001$) respectively (Figures 4.2 and 4.3). Both comparisons show that the samples with the highest number of sprouted grains had the highest amount of α -amylase activity. However three year-location combinations with no visible sprouting and six year-location combinations showing no FDB fluorescence did have measurable amounts of α -amylase activity, indicating an additional major source of α -amylase to be non-sprouting related.

* The adjusted R^2 is the fraction of variation accounted for (R^2) adjusted for degrees of freedom. The adjusted R^2 ($= R^2_{adj}$) will in the text simply be referred to as R^2 . Fractions of variation accounted for, not adjusted for degrees of freedom, are not presented. It should be noted that unlike the unadjusted fraction ($\in \{0,1\}$), R^2_{adj} can technically assume negative values.

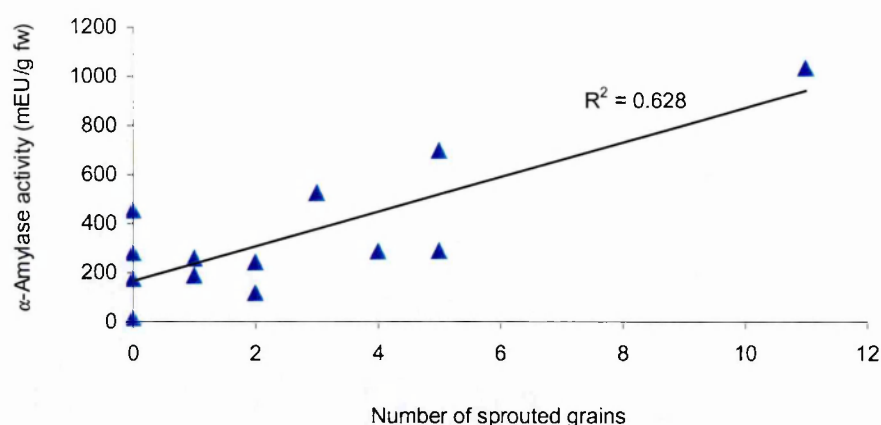


Figure 4.2 Relationship between α -amylase activity and the number of grains visibly sprouted in a 100 grain sample

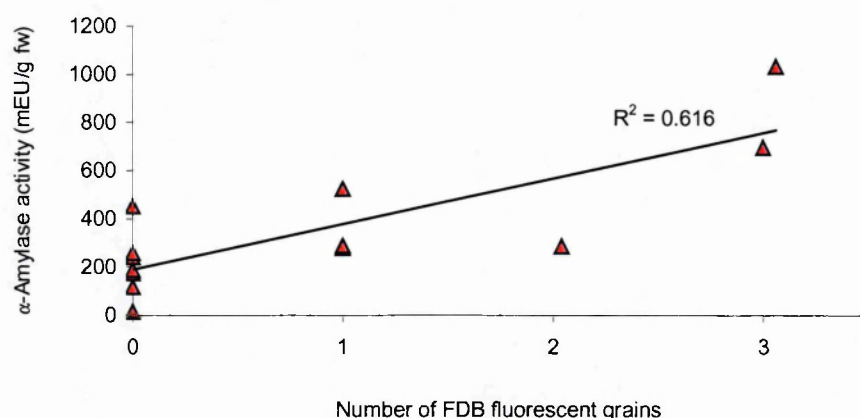


Figure 4.3 Relationship between α -amylase activity and the number of grains showing FDB fluorescence in a 50 grain sample

4.3.4 Weather conditions and enzyme activity

Maximum daily temperature (T_{\max}) measurements taken during the period spanning grain development and ripening period show that the highest temperatures occurred in the first two weeks of August for both 1998 (Figure 4.4) and 1999 (Figure 4.5). In contrast T_{\max} measured in Trumpington 1995 regularly reached above 30°C from the beginning of July (Figures 4.6). T_{\max} averaged for the four trial locations in June and July 1998, the months in which the bulk of the grain development is expected to have taken place (dates in simulated grain development in Table 4.3), only rose above 25°C on the 21st of both

months. Although individual temperature measurements for the four 1999 trial locations exceeded 25 °C in many occasions, mean T_{\max} did not pass 25°C in June, but on two occasions peaked above this value, on 19 and 24/25 of July. The average temperature for the first two weeks of July 1999 was between 3°C and 4°C higher than the average temperature for the first two weeks of July 1998.

AMY2 bands.

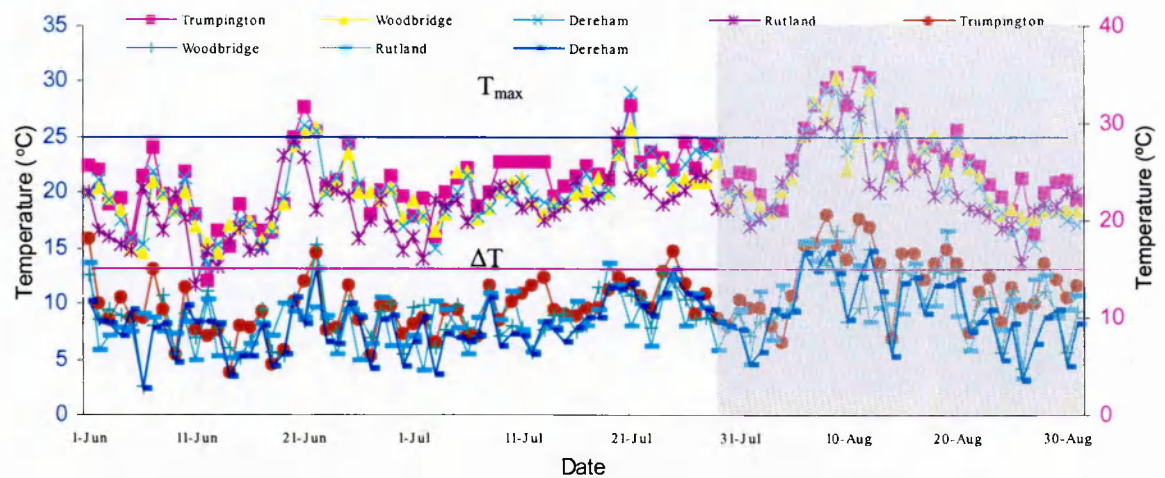


Figure 4.4 Left Y-axis Maximum daily temperature for 1998, ■ Trumpington ▲ Woodbridge × Dereham ✕ Rutland, Right Y-axis associated diurnal temperature difference ● Trumpington | Woodbridge — Dereham — Rutland (grey area falls outside simulated grain development period)

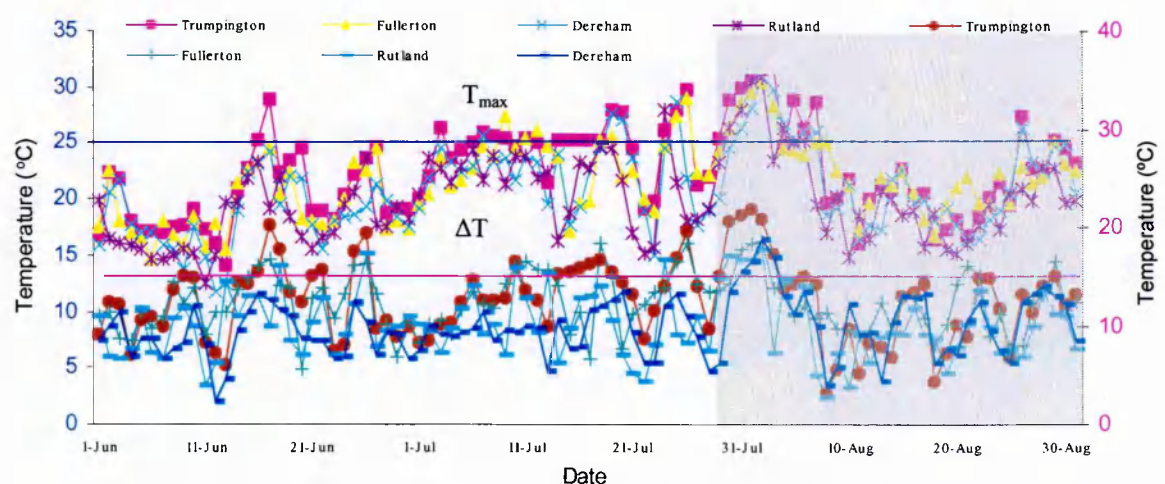


Figure 4.5 Left Y-axis Maximum daily temperature for 1999, ■ Trumpington ▲ Woodbridge × Dereham ✕ Rutland, Right Y-axis associated diurnal temperature difference ● Trumpington | Woodbridge — Dereham — Rutland (grey area falls outside simulated grain development period)

Diurnal temperature difference (ΔT), the positive difference between T_{\max} and T_{\min} , tended to increase as the T_{\max} increased, for both 1998 (Figure 4.4) and 1999 (Figure 4.5).

A comparison of mean T_{\max} and mean ΔT for the four trial locations gave a correlation coefficient of 0.70 for 1998 and 0.67 for 1999, for the recorded time period. A correlation coefficient of 0.85 was found for the single sample from 1995. A high temperature period, 25 July to 4 August, with temperatures peaking above 30°C had a correlation coefficient of 0.91. A similar value of 0.89 was found for the same short period in 1999.

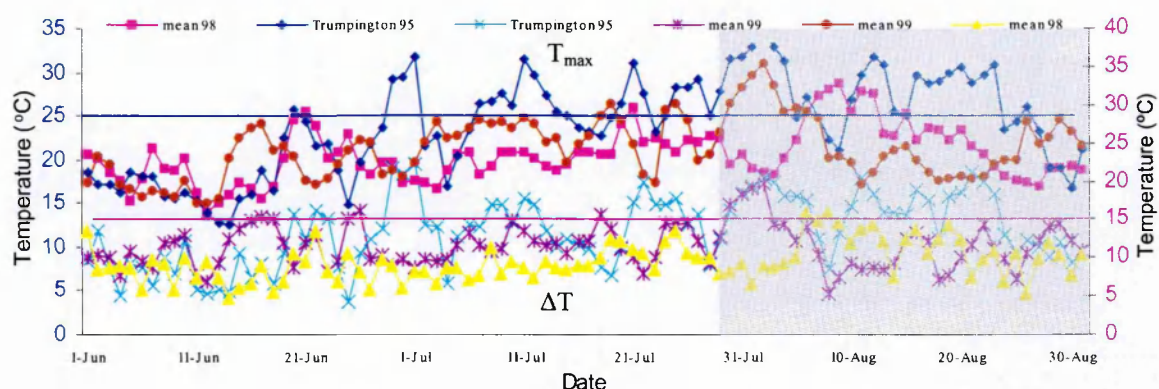


Figure 4.6 Left Y-axis Maximum daily temperature for 1995 and average maximum daily temperature for 1998 and 1999, ■ mean 98 ● mean 99 ♦ Trumpington 95, Right Y-axis associated diurnal temperature difference, ▲ mean 98 ✕ mean 98 ✕ Trumpington 95 (grey area falls outside simulated grain development period)

Only the 1995 Trumpington data showed T_{\max} nearing or exceeding 30°C in all three recorded months (Figure 4.6). In 1998 the mean for ΔT peaked at 13.6°C and 13.5°C on 22 June and 25 July correspondingly. In 1999 the largest differences came a few days later than in 1998, 14.2°C on 26 June and 16.1°C on 31 July.

Table 4.3 Dates in grain development calculated for three years using the AFRC-model

Harvest Year	Anthesis	Beginning of grain filling	End of grain filling	Maturity
1995	26-May	13-Jun	18-Jul	21-Jul
1998	23-May	07-Jun	21-Jul	27-Jul
1999	23-May	06-Jun	16-Jul	20-Jul

Sowing date used in model: 18 September, model temperature: Central England Temperature

Dates at which certain stages in grain development (Table 4.3) were reached were estimated using the AFRC-model (Weir *et al.*, 1984).

A comparison was made in relation to α -amylase activity between meteorological measurements close to two high temperature periods. One ($> 26^{\circ}\text{C}$) occurred around the third week of July in all three years (Figure 4.6) and was near the end of simulated grain development (Table 4.3). A shorter and relatively lesser peak in temperature occurred during early grain development, around the third week of June in all three years.

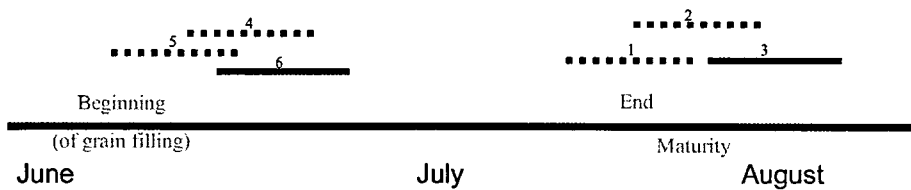


Figure 4.7 Depiction of three 11 day periods during the latter stages of grain development and three 11 day periods during early grain development, for which weather data was examined in detail. Red dotted lines show periods for which both rainfall and temperature were assessed, blue lines show periods for which only rainfall was examined. Simulated dates in grain development are shown in grey.

For the late temperature peak, rainfall and temperature data was accumulated for two 11 day periods, the first period running up to and included the simulated end of grain filling date and the second overlapping period had the end of grain filling as the median. In addition, rainfall was accumulated for a third period, 11 days after simulated end of grain filling (Figure 4.7). The temperature parameters calculated were $\Sigma \Delta T$, $^{\circ}\text{Cdays}$, ΣT_{max} and $\Sigma(T_{\text{max}} - 25)$. $\Sigma(T_{\text{max}} - 25)$ is the accumulation of the positive outcomes for $T_{\text{max}} - 25^{\circ}\text{C}$, in essence the sum of maximum daily temperatures for those days when the T_{max} reached above 25°C .

Table 4.4 Correlations between rainfall and temperature parameters for period 1 and 2, during late grain development

		Accumulated rainfall
Temperature parameters		Period 1
	$\Sigma\Delta T$	0.239
	$^{\circ}\text{Cdays}$	0.601
	ΣT_{max}	0.513
	$\Sigma(T_{\text{max}}-25)$	0.569
		Period 2
	$\Sigma\Delta T$	0.058
	$^{\circ}\text{Cdays}$	0.458
	ΣT_{max}	0.385
	$\Sigma(T_{\text{max}}-25)$	0.629

Weather data accumulated for 11 day periods.

$\Sigma\Delta T$: accumulated diurnal temperature difference,

$^{\circ}\text{Cdays}$ see section 4.2, ΣT_{max} : accumulated maximum

daily temperature, $\Sigma(T_{\text{max}}-25)$: sum of maximum daily

temperatures for those days when T_{max} reached above 25°C.

Weather data collated from 5 weather stations (see section 4.2.2).

A complete correlation matrix compiled for the eight temperature parameters, accumulated rainfall over three periods, and α -amylase measurements, showed that same period correlation coefficients for temperature and rainfall measurements were positive, indicating an increased risk of rainfall as the temperature increased (Table 4.4). All significant correlations with α -amylase measurements were negative, signifying that an increase in temperature, and possibly rainfall, lowered grain α -amylase activity (Table 4.5). The highest number of significant environment-amylase correlations was found in relation to Phadebas gel measurements, with clear PMAA showing significant correlations with three temperature parameters in both period 1 and 2, while the number of grains active on Phadebas gel correlated significantly with three period 2 temperature parameters and with four period 1 temperature parameters.

Of these, the interaction between the number of grains showing α -amylase activity on Phadebas gel and ΣT_{max} for the period leading up to end of grain filling (period 1), had the highest coefficient of correlation (Table 4.5). The relationship between the two parameters

Table 4.5 Correlations for a high temperature period in late grain development of 13 year-location combinations, with environmental parameters in columns and α -amylase measurements in rows

	Temperature parameters								Accumulated rainfall		
	1 st period				2 nd period				Period 1	Period 2	Period 3
	$\Sigma\Delta T$	$^{\circ}\text{Cdays}$	ΣT_{max}	$\Sigma(T_{\text{max}}-25)$	$\Sigma\Delta T$	$^{\circ}\text{Cdays}$	ΣT_{max}	$\Sigma(T_{\text{max}}-25)$			
$\ln \text{mEU/g}$	-0.314	-0.506	-0.470	-0.782	-0.082	-0.509	-0.432	-0.610	-0.317	-0.687	0.126
Active grains*	-0.800	-0.904	-0.916	-0.733	-0.339	-0.863	-0.799	-0.778	-0.408	-0.483	0.625
PMAA^	-0.892	-0.769	-0.846	-0.593	-0.596	-0.698	-0.752	-0.670	-0.282	-0.131	0.500

Correlation coefficient significant ($P < 0.05$) outside interval $\{-0.6664, 0.6664\}$ (Campbell, 1989), figures in red. * Number of grains showing α -amylase activity on Phadebas gel. ^ Number of grains showing clear PMAA discoloration on Phadebas gel. Weather data accumulated for 11 day periods. $\Sigma\Delta T$: accumulated diurnal temperature difference, $^{\circ}\text{Cdays}$ see section 4.2, ΣT_{max} : accumulated maximum daily temperature, $\Sigma(T_{\text{max}}-25)$: sum of maximum daily temperatures for those days when T_{max} reached above 25°C . Weather data collated from 5 weather stations (see section 4.2.2).

is largely linear with 81.5% ($P < 0.01$) of the α -amylase variance accounted for by high temperature (Figure 4.8). The same temperature parameter also exhibited a linear relationship with the number of grains showing clear PMAA on Phadebas gel, producing an R^2 of 67.5% ($P = 0.004$) (Figure 4.9), thus indicating that a rise in peak daytime temperatures can decrease the amount of α -amylase accumulated in harvested grain.

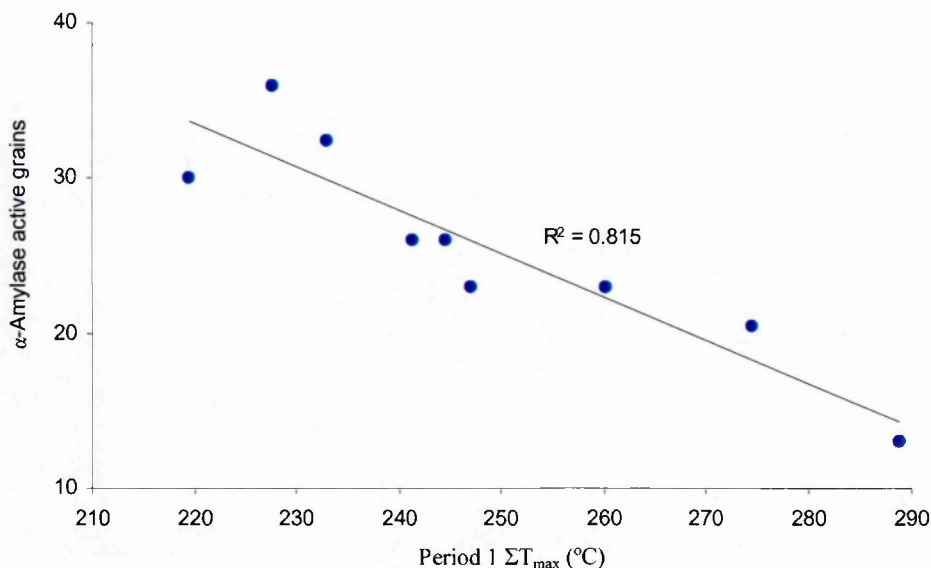


Figure 4.8 Relationship between the number of α -amylase active grains on Phadebas gel and ΣT_{max} , collated for an 11 day period leading up to and including simulated end of grain filling (period 1)

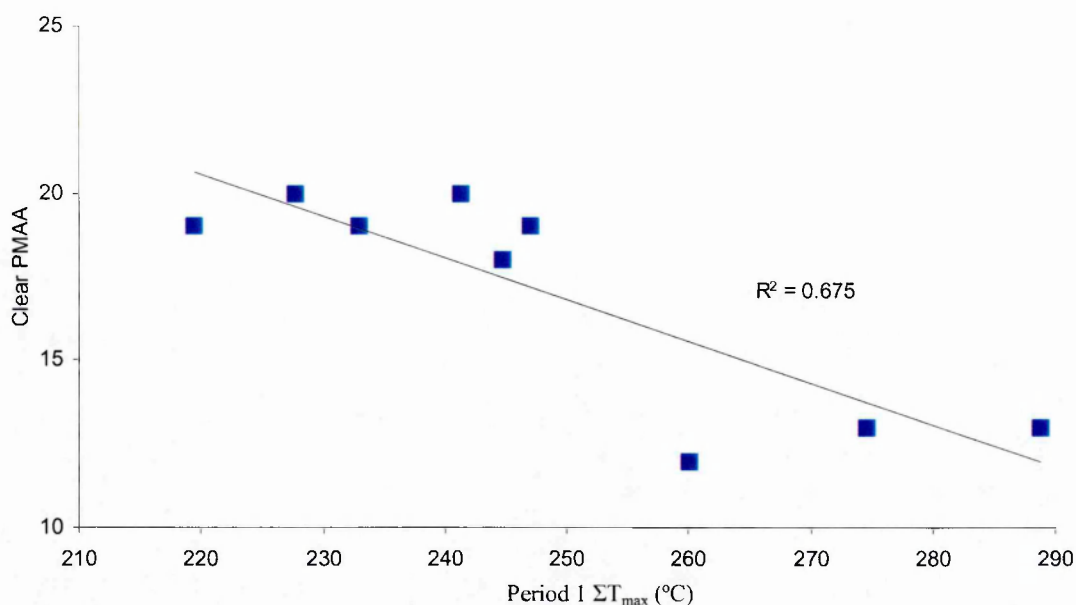


Figure 4.9 Relationship between the number of grains showing clear PMAA on Phadebas gel and ΣT_{max} , collated for an 11 day period leading up to and including simulated end of grain filling (period 1)

The correlation improved when the number of grains showing clear PMAA on Phadebas gel was viewed in relation to the accumulated diurnal temperature difference in period 1. A higher R^2 of 76.7% ($P = 0.001$) also demonstrated a better linear fit (Figure 4.10).

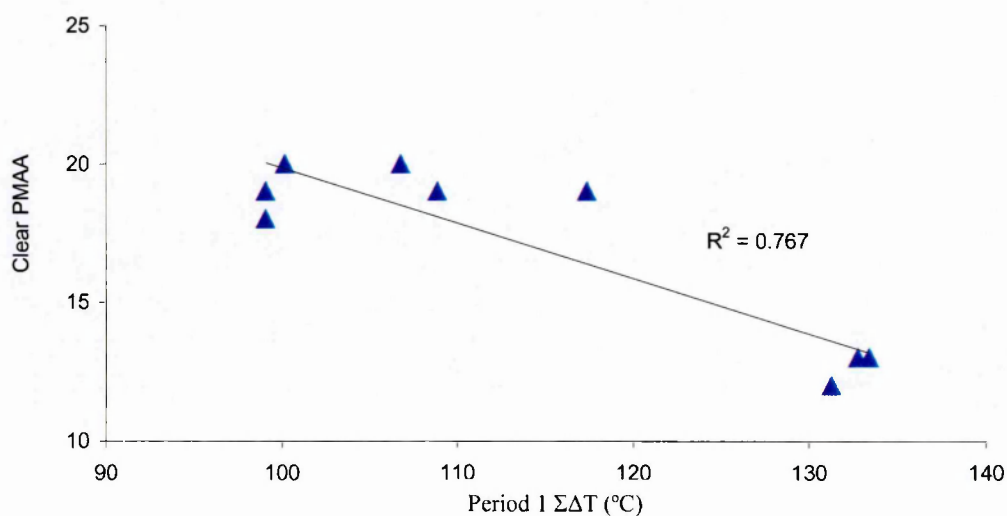


Figure 4.10 Relationship between the number of grains showing clear PMAA on Phadebas gel and $\Sigma \Delta T$, collated for an 11 day period leading up to and including simulated end of grain filling (period 1)

Linear regression analyses mirrored results presented in the correlation matrix (Table 4.5) and showed significant relationships between α -amylase and weather parameters to be of a negative linear nature. This means that by and large increases in temperature, and possibly rainfall, tend to lower α -amylase activity, although in the additional period 3, occurring closer towards harvest, rainfall does appear to increase grain α -amylase.

For the early temperature peak, temperature and rainfall data was again accumulated for two 11 day periods. The first period (period 4) had the date of the temperature peak apex as the median, while the second period (period 5) began on the simulated start of grain filling date. Work by Major (1999) suggests that high humidity, occurring after the PMAA-susceptible grain development stage, might influence PMAA accumulation. Therefore an additional third rainfall period was taken into account, beginning five days after the start of grain filling (period 6) (Figure 4.7). Period 4 °Cdays showed significant positive correlation with accumulated rainfall in period 4 ($r = 0.816$, $P < 0.05$) and period 6 ($r = 0.798$, $P < 0.05$). A negative correlation was found for period 5 $\Sigma(T_{\max} - 25)$ and accumulated rainfall in period 5 ($r = -0.678$, $P < 0.05$). No other temperature-rainfall combinations were found to correlate significantly. While rainfall correlated positively with Phadebas gel measurements, no significant relationship was found with sample α -amylase activity (Table 4.6).

Surprisingly both period 4 and 5 $\Sigma \Delta T$ and period 5 ΣT_{\max} correlated negatively ($P < 0.05$) with the number of clear PMAA grains on Phadebas gel, indicating that an increase in these temperature parameters during early grain development lowers PMAA. In fact, all but one correlation between temperature parameters and α -amylase measurements was negative, signifying that early temperature increases lower grain α -amylase in general. The relationship between period 5 $\Sigma \Delta T$ and the number of grains showing clear PMAA on

Table 4.6 Correlations for a high temperature peak during early grain development of 13 year-location combinations, with environmental parameters in columns and α -amylase measurements in rows

	Temperature parameters								Accumulated rainfall		
	4 th period				5 th period				Period 4	Period 5	Period 6
	$\Sigma\Delta T$	$^{\circ}\text{Cdays}$	ΣT_{max}	$\Sigma(T_{\text{max}}-25)$	$\Sigma\Delta T$	$^{\circ}\text{Cdays}$	ΣT_{max}	$\Sigma(T_{\text{max}}-25)$			
Ln mEU/g	0.216	-0.093	0.042	-0.065	0.091	-0.670	-0.436	-0.853	0.080	0.453	0.226
Active grains*	-0.448	0.392	0.094	-0.174	-0.581	-0.348	-0.615	-0.764	0.696	0.819	0.783
PMAA^	-0.742	0.338	-0.151	-0.146	-0.864	-0.194	-0.675	-0.579	0.668	0.646	0.725

Correlation coefficient significant ($P < 0.05$) outside interval $\{-0.6664, 0.6664\}$ (Campbell, 1989), figures in red. * Number of grains showing α -amylase activity on Phadebas gel. ^ Number of grains showing clear PMAA discoloration on Phadebas gel. Weather data accumulated for 11 day periods. $\Sigma\Delta T$: accumulated diurnal temperature difference, $^{\circ}\text{Cdays}$ see section 4.2, ΣT_{max} : accumulated maximum daily temperature, $\Sigma(T_{\text{max}}-25)$: sum of maximum daily temperatures for those days when T_{max} reached above 25°C . Weather data collated from 5 weather stations (see section 4.2.2).

Phadebas gel was largely linear with 71.1% ($P = 0.003$) of the α -amylase variance accounted for by this temperature parameter (Figure 4.11). The large correlation coefficient for period 5 $\Sigma(T_{\text{max}} - 25)$ and the natural logarithm of sample α -amylase activity (Table 4.6) needs to be viewed with some caution as only two out of nine temperature related observations were of a value other than 0.

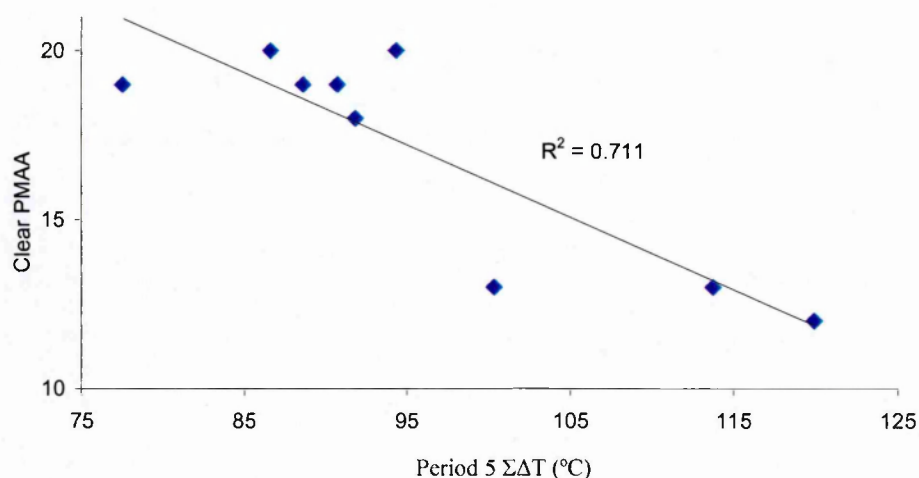


Figure 4.11 Relationship between the number of grains showing clear PMAA on Phadebas gel and period 5 $\Sigma\Delta T$

Multiple linear regression analysis, taking both temperature and rainfall parameters into account, produced one significant regression ($R^2 = 0.686$, $P = 0.013$) for the number of

grains showing clear PMAA on Phadebas gel, when period 5 ΣT_{\max} and rainfall accumulated for period 6 were selected as the explanatory variates (Figure 4.12). A decrease in PMAA incidence and reduced rainfall tended to accompany an increase in daily maximum temperature ($z_{(\text{PMAA})} = 102 \times 10^{-3} x_{(\text{rainfall})} - 156 \times 10^{-3} y_{(\text{temperature})} + 44.6$). While not significant ($r = -0.285$), a negative trend between the two explanatory variates was found, indicating that an increase in maximum daily temperature could lower precipitation.

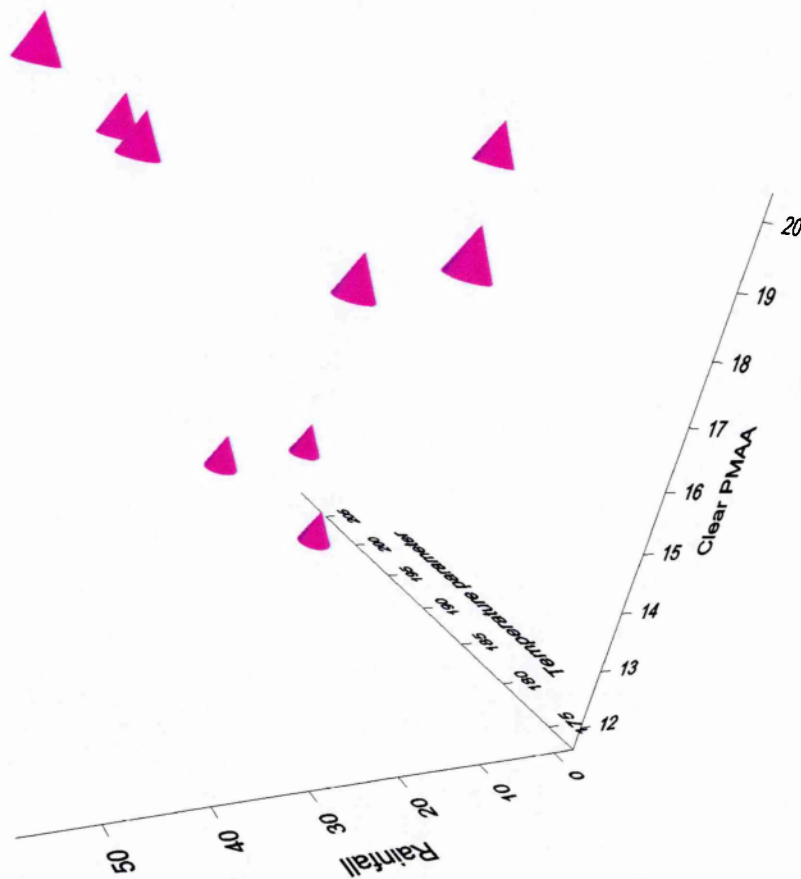


Figure 4.12 Relationship between the number of grains showing clear PMAA on Phadebas gel and period 5 ΣT_{\max} ($^{\circ}\text{C}$) and rainfall (mm) accumulated for period 6, with smaller symbols indicating greater depth of image

Multiple linear regression analysis with sample α -amylase activity did not produce any significant regressions.

4.4 Discussion

There is a discrepancy in the results, since the Phadebas gel test showed only one sample to have PMAA as the sole source of α -amylase activity in the grain, but the results from esterase/lipase fluorescence and AMY2 iso-electric focussing assays revealed at least four year-location combinations with activities presumably due to PMAA as no considerable amount of fluorescence or AMY2 activity were found, indicating a lack of substantial sprouting. It is possible that a higher number of grains analysed would have resolved this discrepancy. A larger sample size scored for visual sprouting and FDB fluorescence would in all probability have shown the presence of a small number of sprouted grains in the samples, producing only minimal amounts of AMY2 and thus not forming any significant or detectable bands on IEF gels. The possibility exists that the number of clear PMAA grains counted was lower than the actual number and that the number of grains scored as sprouted was larger than in reality, as migration of enzyme on the Phadebas gel in some instances produced irregular discoloration patterns. It is assumed that these patterns are not due to pericarp α -amylase as this is not easily detectable on Phadebas gel (Major, 1999). However, without a closer inspection of the isozyme composition, contribution by pericarp α -amylase to halo formation on Phadebas gel can not be fully ruled out. It should be recognised that the Phadebas gel test is limited in its ability to discriminate between PMAA and sprouting. Grains with both sources of α -amylase will not be distinguishable from grains with only sprouting as the source of α -amylase, especially when sprouting is severe. PMAA could, in theory, be distinguished from incipient sprouting in longitudinal sections of grain incubated on Phadebas gel. PMAA would appear as a ribbon of discoloration covering the full length of the grain (Cornford *et al.*, 1987b), while incipient sprouting would be seen as discoloration next to the scutellum (section 5.3.2.2; Cornford *et al.*, 1987b). However, Major (1999) found that the technique gave a higher resolution in

general when using transverse sections of grain. In transverse sections, scutellar α -amylase, and subsequently triggered aleurone α -amylase, was often first noticed in the dorsal area of the grain (Plate 4.1), as the scutellum tends to tilt inward on the dorsal side. This observation however depends on the distance between the scutellum and the position of the cut. The fact that in many cases dorsal activity is followed by a noticeable increase in discoloration in the crease might be due to two parts of the aleurone layer being in close proximity to each other, effectively doubling local α -amylase secretion. It is possible that in some instances incipient sprouting can be mistaken for PMAA.

Major *et al.* (1996) reported that a controlled high temperature period peaking at 26°C during early grain development would induce PMAA in several winter wheat varieties. All Rialto samples showed PMAA formation to some degree. Average T_{\max} for June 1998 and 1999 did not exceed 26°C, but did exceed this temperature on 21 July 1998 (simulated end of grain filling 21 July) and twice on 19 and 25 July 1999 (simulated end of grain filling 16 July) (Figure 4.6). These late high temperature (>26°C) periods would have occurred after the most susceptible period (Major *et al.*, 1996) in grain development. As a result, the increased temperature appears to lower grain α -amylase activity, most likely through a reduction in precipitation, although no significant negative correlation was found between temperature in a certain period and rainfall in subsequent periods. An earlier June high temperature peak occurring during simulated early grain development was a more suitable candidate as a trigger for PMAA formation. However, regression analysis showed this not to be the case, as high temperature tended to reduce α -amylase activity. Grains developing during this period did appear to be more susceptible to rainfall, and probably to the accompanying high humidity, producing greater crease α -amylase activity (Table 4.6). As there was no significant correlation with sample α -amylase activity, but positive correlations were found for the number of grains showing clear PMAA on Phadebas gel,

early rainfall appears to modify grain α -amylase activity, not through the induction of pre-harvest sprouting, but through its effect on PMAA expression. It should be noted that because of the limited data available the AFRC-model (Weir *et al.*, 1984), used to simulate grain development, only has a limited predictive value. Even so, these results leave open for debate, a possible difference in varietal response, between cv. Rialto and older varieties used in earlier PMAA studies, or the existence of a time/temperature cumulative mechanism, at work at lower temperatures compared to the high temperature used by Major (1999). Major (1999) reported that in his studies the length of the high temperature treatment during early grain development did not have a significant effect on α -amylase activity, when using a 26°C daytime temperature. Work by Norman *et al.* (1982) carried out in a laboratory environment suggests the existence of a threshold temperature, below which prolonged exposure to the sub-threshold temperature will not increase aleurone sensitivity to gibberellic acid. The large percentage of correlation between T_{\max} and ΔT , found for all three years in which Rialto samples were collected, shows that increases in daytime temperature during grain development are not matched by increases in minimum night-time temperature during that period. In addition to the requirement for exposure to an elevated temperature, Norman *et al.* (1982) found that in their laboratory experiments the aleurone must also be subjected to a short low temperature treatment of a few seconds, effectively rapidly lowering the aleurone temperature by just a few degrees. Major (1999) used a relatively high night-time temperature of 20°C during a three day high temperature treatment, in order to induce PMAA. This suggests that the large diurnal difference in temperature found in the field is not responsible for the temperature shock *in vivo*, Norman *et al.* (1982) suggest is essential for triggering aleurone α -amylase secretion.

Although at first sight, α -amylase activity of Rialto samples seems to be higher in 1999 compared to 1998 (Figure 4.1), closer analysis failed to show a significant difference

between amylase activities of samples taken in 1998 and samples taken in 1999. It is possible that this result is due to the relatively small number of samples available for these years separately. Rosell *et al.* (2002) examined enzyme activity for the 1999 crop of several Spanish bread wheats including the high Hagberg variety Soissons. Using an adaptation (Sirou *et al.*, 1990) of the method by McCleary and Sheehan (1987) and redefined test conditions (Rosell *et al.*, 2001) they found α -amylase activities of 170 to 758 mmol PNP h⁻¹ g⁻¹ fw in undamaged grain and approximately 254 mmol PNP h⁻¹ g⁻¹ fw for cv. Soissons in particular. Assuming that their test conditions are comparable to those of the previous authors, the enzyme activities found appear lower, but not dissimilar to amylase activity in a sample from the non-sprouted 1995 Rialto crop grown in the UK. Rosell *et al.* (2002) found an accompanying falling number of 349 s for Soissons. Equally in the UK, Soissons produces relatively high HFNs, 312 s in 1998 (Anonymous, 1998) and 317 s in 1999 (Anonymous, 1999). The 1995 Rialto crop showed no signs of visible sprouting and did not show any FDB florescence, but did show clear PMAA activity in 26% of the grain. In addition, although the amount of α -amylase in the Rialto samples varied between 10 and 1030 mEU/g, in samples with α -amylase identified as mainly due to PMAA by exclusion of sprouting as a major amylase source (using visual and FDB assessments), activity did not exceed 450 mEU/g. This could support the notion that in low Hagberg years, deterioration of α -amylase activity in cv. Rialto is likely due to a combination of α -amylase sources in the grain. Lunn *et al.* (2001a) found that PMAA and pre-maturity sprouting were not only more common than expected in four UK winter wheats, but that the most usual pattern was for α -amylase to accumulate by several modes. In addition to the effect of α -AMY1 Lunn *et al.* (2001b) proposed that pericarp derived α -AMY2 can facilitate lowering of HFN in some UK wheat crops. However, Major *et al.* (1996) demonstrated that the potential for high α -amylase in harvested grain due solely to PMAA does exist. Their high temperature treatment applied in a controlled environment

was similar to conditions observed earlier in the field during grain development (Major, 1999). Not having identified any signs of sprouting or α -AMY2, they measured α -amylase activities of up to 878 and 907 mEU/g dry weight for the varieties Pastiche and Riband respectively.

4.5 Conclusion

It has been shown that some of the α -amylase activity detected in samples of cv. Rialto, collected in different years and from several different locations, is due to pre-maturity α -amylase activity. This provides strong support for the hypothesis that the winter wheat variety Rialto is PMAA-susceptible. In addition, although previous studies carried out in controlled environments have identified a high temperature period in early grain development, followed by slow grain drying, as an environmental trigger for PMAA, the present study clearly demonstrated the potential of transient high temperatures, in early grain development, to reduce grain α -amylase formation.

5 The induction of pre-maturity α -amylase activity in controlled environment grown plants

5.1 Introduction

Work on PMAA in winter wheat and PMAA related LMA (section 2.2.2) in Australian spring wheat has demonstrated the potential of this problem with excess hydrolytic enzyme to reduce the breadmaking quality of flour (Mrva and Mares, 1996a; Major, 1999).

Detailed analysis of PMAA and its environmental stimuli in the field is however fraught with complexity, as the lack of control exerted over the plant's environment means that any clues as to a trigger for PMAA formation are confounded by or hidden in noise.

When Major (1999) looked in to the environmental requirements for induction of PMAA he used plants grown in the glasshouse through to anthesis. At anthesis, plants were moved to a controlled environment (CE) cabinet, where a temperature treatment was applied. To ensure that varying growing conditions, as found for glasshouse-grown material, did not confound PMAA induction in the current study, plants were grown fully in CE. Major (1999) reported a specific three day high temperature treatment during early grain development to be a stimulus for high PMAA synthesis in some older winter wheat varieties. In the current study, however, working with field grown material of the newer variety Rialto (chapter 4), it was found that a transient high temperature period in early grain development lowered PMAA, although analysis was carried out on a limited number of samples.

It has nevertheless been shown (see chapter 4) that a more recent variety like Rialto is still PMAA susceptible, and that PMAA thus remains in present crossing programs of UK wheat breeders. In addition to cv. Rialto, two other UK winter wheat varieties were chosen as test varieties in full CE experiments because of their Hagberg characteristics (section 5.2.1). Spread out over nine CE experiments an assessment was to be made of (a) the effect of grain development stage during high temperature treatment, (b) the effect of temperature fluctuation during high temperature treatment and (c) the effect of the duration of the high temperature treatment on PMAA expression. In addition, the experiment would also give information on (d) the effect of slow grain drying and (e) the effect of genetic background and (f) Rht-alleles on the amount of PMAA produced. Not all experiments could however be carried out in their entirety during this project (see section 5.2.4).

Taken together, the experiments tested the hypothesis that a specific high temperature treatment during a particular receptive stage in grain development, followed by slow grain drying, promotes PMAA formation in susceptible UK winter wheat varieties.

Of the four experiments fully analysed (Table 5.1), the first, designated CE HA 1, tested the hypothesis that there is a difference in PMAA susceptibility between milk and dough grain development stages, when using an earlier established three day high temperature treatment (Major, 1999). Because the results from CE HA 1 were initially greeted with some suspicion as a fungal infection was found to be present in both vegetative tissues and grains, a third experiment was to take a step back. Experiment CE HA 3 would examine the effect of the established three day high temperature treatment on fully CE grown plants. Major (1999) had used glasshouse plants for determining the effectiveness of the high temperature treatment. CE HA 3 was set up to test the hypothesis that the specific high temperature treatment during early grain development could stimulate PMAA

formation, in fully CE grown UK winter wheat varieties. The CE HA 6 experiment was set up to test the hypothesis that a high temperature treatment during early grain development followed by slow grain drying could stimulate PMAA formation. As Major (1999) transferred plants to the high temperature treatment from a relatively cold temperature regime, experiment CE HA 8 investigated the hypothesis that a change in diurnal temperature difference (see results section 4.3.4) combined with a high temperature treatment stimulates PMAA formation. Norman *et al.* (1982) have shown that under laboratory conditions, in addition to a high temperature treatment, there is a requirement for a temperature shock in order for excessive α -amylase to be formed in de-embryonated grains. It is reasoned that this shock could translate to an increase in the daily temperature fluctuation in the field. Weather data for 10 site/year combinations (chapter 4) suggests that during some high temperature periods, in UK summers, there is no apparent change in the daily minimum temperature (T_{\min}), while daily maximum temperature (T_{\max}) peaks well above average.

5.2 Materials and methods

5.2.1 Varieties

In addition to Rialto, two other PBIC winter wheat varieties, Haven and Option, were chosen for full CE experiments because of their difference in HFN and sprouting scores. Haven has a low to medium HFN score (Anonymous, 1995 and 1996), while Rialto (Anonymous, 2000 and 2001) and Option (Anonymous, 2002 and 2003) have a medium (249 s adjusted to 220 s a year later) and high (288 s adjusted to 277 s a year later) HFN respectively. A Hagberg of 190 to 219 s is considered to fall within the low HFN category, 220 to 249 s scores as medium and a HFN of 250 to 279 s is classified as high

(Anonymous, 1995). Haven (Anonymous, 1995 and 1996) has a sprouting resistance score of 2 compared to 6 for Rialto (Anonymous, 2000 and 2001) and 8 for Option (Anonymous, 2002).

5.2.2 Growing plants in a controlled environment

Prior to sowing, grains were given a micro dressing containing carboxin and thiram (Anchor, Uniroyal, Evesham, Worcestershire) at 3 $\mu\text{l/g}$ grain ($< 16\%$ moisture), to protect young plants against foot rot diseases especially during vernalisation (see section 5.2.3). Grain was placed in a large skirted centrifuge tube with screw cap and securely fixed to a vertical shaker. At medium speed, the correct amount of dressing diluted by 50-100% of its volume with distilled water, was slowly added using a micro pipetter (20 μl maximum). The centrifuge tube was then sealed with the screw cap and grains shaken at higher speed, making sure that the grains were not damaged or fractured. Grains were sown as soon as possible.

Grains were sown in trays in John Innes No 2 (Keith Singleton's Seaview Nurseries, Egremont, Cumbria) compost and germinated in a controlled environment cabinet (Fitotron, Sanyo Gallenkamp plc., UK) or in a growth cabinet (Sanyo Environmental Test Chamber, Sanyo Gallenkamp plc., UK) (Plate 5.1) under a 15°C daytime and 10°C night-time temperature regime. The photo period was limited to 10 h each day, consisting of full fluorescent lighting only. Around Zadoks' growth stage 12 (see last paragraph in this section) seedlings were moved to a fridge maintained at approximately 4°C for vernalisation. The plants were given an 8 h light period provided by four to six fluorescent tubes. Plants were vernalised over a period of 60 days.

After vernalisation, individual plants were transplanted into pots with a 10 cm diameter, using John Innes No 2 compost, and placed in a controlled environment cabinet (Fitotron, Sanyo Gallenkamp plc., UK or Conviron, Controlled Environments Ltd., Canada) (Plate 5.1). The floor size of two smaller Fitotrons limited the number of plants to a maximum of about 50 per cabinet, while the Conviron would accommodate a maximum of 60 plants. A larger Fitotron could hold up to 65 plants. Due to practical reasons, especially to ensure that plants were not damaged, fewer plants than the maximum number possible were placed in the cabinets. For the first three experiments (see section 5.2.4) the cabinets were maintained at 15°C during a day period of 12 h, and at 10°C during the dark period, for the first 30 days. Relative humidity was set to 70%. After 30 days the temperature regime was changed to 20°C during a 16 h day period, and 15°C during the dark period. In subsequent CE experiments plants were moved directly into a 20/15°C day-night regime. This was done to reduce the length of time it took for plants to reach anthesis. Earlier tests had shown that a direct shift to a 20/15°C regime had no apparent adverse effects on vegetative plant development.

Plant growth and grain development in particular was monitored using the ZGS score, a method for visual assessment of plant development stage (Zadoks *et al.*, 1974; Tottman and Makepeace, 1979; Tottman, 1987). The principal Zadoks' growth stages are Germination (0), Seedling growth (1), Tillering (2), Stem elongation (3), Booting (4), Inflorescence emergence (5), Anthesis (6), Milk development (7), Dough development (8) and Ripening (9) (Tottman, 1987). Each principal growth stage is again split into 10 secondary growth stages, thus the whole of plant development, from sowing through to harvest, can be expressed in 100 steps (ZGS 00-99).

ZGS grain assessment was carried out for three grains in the mid-ear in either position 1, the basal floret, or position 2, the second floret from the base. With regard to PMAA induction in the grain, two periods in grain development were of most interest during this project. These were milk development stage (ZGS 70-79) and the dough development stage (ZGS 80-89). When some modern UK winter wheats, but Rialto in particular, are grown in a glasshouse environment or CE, the beginning of anthesis (ZGS 61) does not correlate well with the emergence of the first anthers, to such an extent, that some ears can contain well-developed caryopses, while no anthers are visible. A reliable estimate of grain development expressed in day after anthesis could therefore not be made. However, in order to acquire an additional indicator of grain development a single mid-ear grain from either position 1 or 2 was removed to determine its moisture content. This was carried out from the second CE experiment onwards (results in section 5.3.3). The moisture content will be presented as moisture as a percentage of fresh grain weight (fw), $(\frac{fw-dw}{fw}) \times 100\%$. Dry weight (dw) was determined using the freeze drying method (section 5.2.3). Depending on the experimental set up, assessment of grain development stage on plants was carried out on a maximum of five most developed tillers. The tillers of interest were tagged at full ear emergence (ZGS 59) and given individual codes.

5.2.2.1 Agronomy

Before and during vernalisation, seedlings were watered with tap water using an ordinary watering can. Plants growing in CE cabinets in individual pots were watered daily with tap water, or as needed, through a narrow hose at ground level. This was done to avoid wetting the leaves and to reduce splash, thus minimising the risk of disease taking hold in the crop. Towards the latter part of vegetative growth, senescence set in amongst the lower leaves and some smaller adventitious tillers. These leaves and tillers were removed to allow for a

better circulation of air. Between experiments, CE cabinets were given a crude decontamination by washing the inside walls with warm soapy water and leaving the cabinets to run empty for five days at 30-35°C and > 90% RH.

During the vegetative growth of test plants growing in 10 cm pots in both glasshouse and CE cabinets, signs were noticed of a nitrogen deficiency (Wallace, 1951). Between the end of stem elongation and ear emergence plants grown for CE experiments were given approximately 2 g of nitrogen fertiliser in granular form (Extran 34.5% N, Hydro Agri (UK) Ltd.).

After experiment CE HA 2 (see section 5.2.4), fungicides were applied prophylactically during the vegetative growth stages. Fenpropimorph (Corbel, BASF plc.) and fenpropidin (Tern, Novartis Crop Protection UK Ltd.) were used against powdery mildew (*Erysiphe graminis*) (Gair *et al.*, 1991) during late seedling and stem elongation stages respectively. Tebuconazole (Folicur, Bayer plc.) was applied twice against foot rot, during stem elongation and ear emergence. During the vegetative growth of CE HA 5 a visible infection by *Septoria* developed on the leaves (Gair *et al.*, 1991; Ballance *et al.*, 1999) of plants in some of the CE cabinets. Plants in this and subsequent experiments were therefore also treated with epoxiconazole (Opus, BASF plc.), as tests on additional infected plants showed that epoxiconazole was effective against the *Septoria* sp(p). causing the symptoms. All fungicide sprays were applied at maximum individual dosage, in a specially designated spraying area outside of the CE cabinets. Plants were returned to the CE when leaves were dry.

5.2.2.2 Maintaining controlled environment

Three different types of CE cabinet were available at Harper Adams. A total of three Convirons (Plate 5.1, top right), two Fitotrons (top left) and one larger version of the latter (bottom right) had to be shared between the various research projects carried out at Harper Adams.

Plate 5.1 CE and growth cabinets at Harper Adams:
Top left small Fitotron, *Top right* Conviron, *Bottom left* Test Chamber, *Bottom right* large Fitotron



Manufacturers: *Top right* Controlled Environments Ltd., Canada
Top left & below Sanyo Gallenkamp plc., UK

Lighting in the smaller Fitotrons was provided by twelve 36 W fluorescent tubes (Osram or Philips) and four 60 W incandescent lamps (Sylvania). The larger Fitotron was equipped

with twenty 50 W fluorescent tubes (Osram or Philips) and eight 60 W incandescent lamps (Sylvania). The Conviron incorporated twelve 115 W high output fluorescent tubes (VHO fluorescent tubes, Sylvania) and nine 25 W Edison screw light bulbs (Osram or Philips).

Light intensity was measured at approximate ear height using a quantum sensor (Delta-T, Delta-T Devices Ltd., Cambridge, Cambridgeshire) and a millivolt meter (AVO M2036, RS Components Ltd., Corby, Northamptonshire) and was expressed as the quantum flux density within the photosynthetically active 400-700 nm range. The PAR flux varied between 300 and 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ depending on the ambient temperature outside the cabinets. Prior to the start of the experiments, PAR measurements in the Fitotrons did not exceed 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$, while maximum total flux should have been 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ according to the manufacturer's specifications. This was determined to be due to an insufficient volume of air being moved through the light-box during peak daytime temperatures and the subsequent overheating and degradation of the fluorescent tubes. The problem was overcome when Sanyo Gallenkamp plc. installed variable speed fans slaved to temperature probes, in the light boxes. The near optimum temperature for the Fitotron light box was determined by Sanyo Gallenkamp plc. to be 28°C. A similar problem was found with the Convirons. Tests at Harper Adams showed the optimal light box temperature in the Convirons ($> 20^\circ\text{C}$) to probably be lower than that of the Fitotron (Figure 5.1). The high temperature problem in the Conviron was overcome to some degree by sealing a simple heat exchanger built into the light box cover and by inserting three adjustable vents into the cover, allowing a larger and predetermined amount of hot air to directly exit the light box.

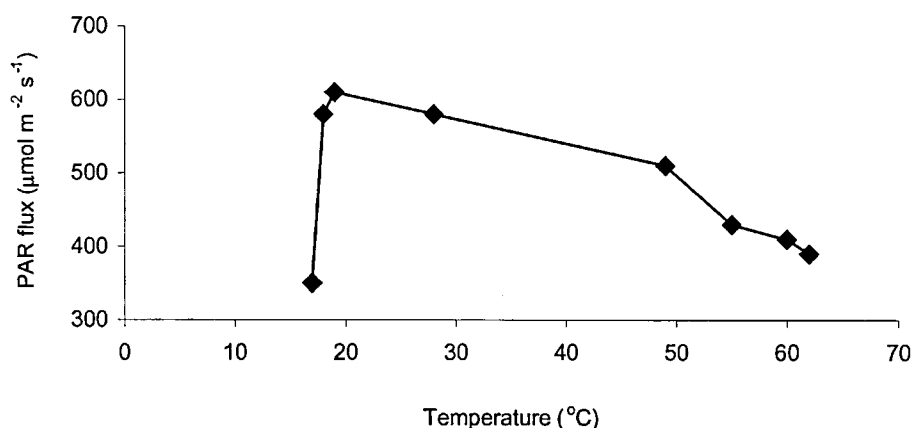


Figure 5.1 PAR flux at approximate ear height and light box temperatures in a Convicon (Controlled Environments Ltd., Canada) CE cabinet

As both humidifier and humidity sensor in the Convicons were not as reliable as those in the Fitotrons, relative humidity was checked and corrected against the measurements given by a humidity sensor (SKH 2010, Skye instruments, Powys, Wales) connected to a data logger (Squirrel logger 1200 Series, Grant Instruments (Cambridge) Ltd., Shepreth, Cambridgeshire).

Temperature inside the CE cabinets was monitored using a temperature logger (Tinytag Plus, Gemini Data Loggers, Chichester, West Sussex). The range of the ambient temperature, outside the cabinets, was measured using a standard minimum-maximum thermometer, reset daily. Cabinet high temperature limits were set to 10°C above the programmed temperature for the light period and the low temperature cut out point was set to 5°C below the programmed temperature for the dark period.

5.2.3 Grain analysis

Total α -amylase measurements (chapter 3) were standardised within and between experiments by expressing enzyme activity per unit grain dry mass. The number of

replications for each experiment is given in section 5.2.4. α -Amylase extractions were analysed in duplicate (chapter 3). Dry mass was determined using a freeze drier (Edwards, Crawley, West Sussex). Grains, frozen to -20°C , were placed in the freeze drier and air from the chamber was evacuated, after the temperature in the freezer reached $< -40^{\circ}\text{C}$. Vacuum was maintained within manufacturer's guidelines. Grains were removed after 24 h and immediately processed further or stored airtight at -20°C with silica gel (Sigma-Aldrich). Grain moisture content was subsequently calculated using the formula in section 5.2.2.

Visual assessment of sprouting, FDB test, α -amylase IEF, Phadebas gel test and total α -amylase activity measurements were carried out as described in section 3.2. Phadebas gel tests were also done on grain not derived from CE experiments (section 5.3.2.2). Grains were incubated either as embryoless transversely cut half grains, or longitudinal half grains. Of transversely cut grains, 20 to 25 grains were incubated per gel dish, of 24, 48 and 72 h sprouted cv. Malacca and Option. Two periods of incubation were employed, 1 and 3.5 h. Similar test, using the same varieties, were done on Phadebas gel, with longitudinal half grains. However, the number of grains per dish was limited to eight and only a one hour incubation was used.

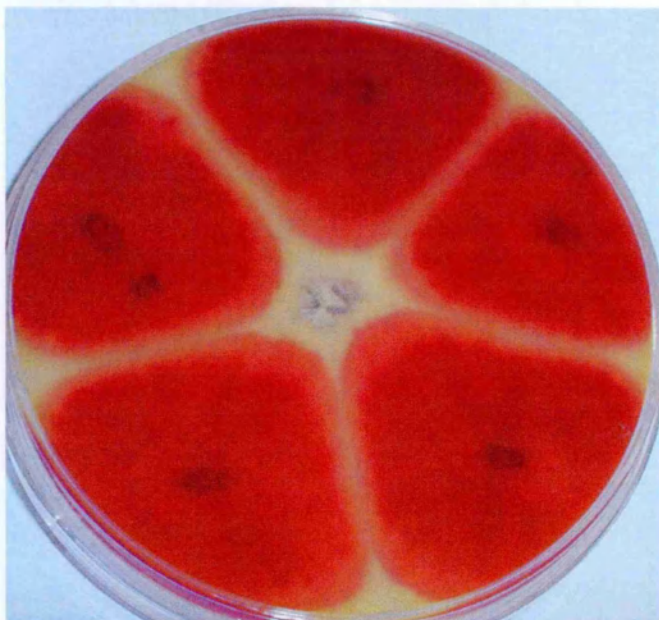
During the first CE experiment, plants were found to be affected by what appeared to be foot rot after an interruption in the operation of the CE cabinet. The interruption happened when most plants were at the ear emergence stage. In order to select a suitable fungicide (see section 5.2.2) to be used in subsequent experiments, grains from affected tillers were surface-sterilised and pathogens isolated from the grain for identification purposes. Grains were immersed in 70% ethanol and immediately whirl-mixed for 2 s. Sterilisation was carried out using a sodium hypochlorite solution (Sigma-Aldrich) with an additive to

reduce surface tension. Sodium hypochlorite was removed by rinsing the grains three times in sterile distilled water. Washed grains were placed crease down on an agar gel. The procedure was carried out in a horizontal laminar airflow cabinet.

As it was suspected that the foot rot was largely caused by pathogens from the *Fusarium* 'foot rot' group (including *Microdochium*), isolation (Brayford, 1997) was carried out on Potato Dextrose Agar (PDA) (DIFCO Laboratories or Merck) with or without streptomycin, in 90 mm disposable vented Petri dishes. Streptomycin (Sigma-Aldrich) was added (10 ml/l) filter sterile (Millipore (UK) Ltd., Watford, Hertfordshire) from a 6.86×10^{-3} M (10 g/l) stock solution to autoclaved PDA after the medium had cooled down sufficiently, but was still liquid. After several days, wads (3 mm) of PDA containing fungal mycelium were taken and re-isolated on PDA with streptomycin for further identification. Petri dishes were exposed to diffuse sunlight, but kept away from direct sunlight, or were placed in an incubator containing a UV light source and maintained at room temperature. A reddish colour dispersing through the mycelium colonised agar (Plate 5.2) indicated the presence of the 'foot rot' pathogens.

Microscopical analysis of conidia (Booth, 1971) produced during sporulation identified the presence of at least two *Fusarium* spp., with definite confirmation of *Fusarium avenaceum*.

Plate 5.2 Fusarium on PDA, isolated from surface sterilised wheat grains



Five surface sterilised grains/Petri dish were placed crease down on agar and exposed to diffuse sunlight, or placed in an incubator with UV source at room temperature. In the absence of the pathogen, grains produced no discoloration of the yellowish agar based gel.

5.2.4 Experimental design

All experiments done in CE were of a randomised blocked design with a minimum of three repetitions. During the treatment phase, plants were divided between CE cabinets of the same manufacturer and if possible, using the same model (Plate 5.1). This was done to ensure the highest possible similarity between background environments, consisting of those factors not varied as part of the experimental set up. Control plants for all experiments were kept in a 20°C 16 h daytime and 15°C night-time temperature regime with a RH of 70%, throughout the grain development stage. Due to the fact that an experiment ran for a relatively long period of time, from sowing the grain through to analysis of the harvested grain, experiments were made to overlap. Grains were sown for vernalisation in general every three to four months. In all, nine experiments were set up to test various hypotheses relating to PMAA formation (Table 5.1).

Table 5.1 Particulars of the nine CE HA experiments carried out with whole plants

	Varieties	Development stage at treatment	High temperature treatment	Post-treatment RH
CE HA 1	Haven, Option, Rialto	milk development dough development	26°C/20°C	85%
CE HA 2	7 winter wheat varieties and one spring wheat			
CE HA 3	Haven, Option, Rialto	milk development	26°C/20°C	85%
CE HA 4	Haven, Option, Rialto			
CE HA 5	Haven, Option, Rialto			
CE HA 6	Haven, Option, Rialto	milk development	26°C/20°C	70% 85%
CE HA 7	Haven, Option, Rialto			
CE HA 8	Rialto	milk development	26°C/20°C 26°C/10°C	85%
CE HA 9*	Rht Maris Huntsman NILs	milk development	26°C/20°C	85%

Failed experiments have no information on treatment shown. Control & post-treatment temperature regime was 20°C/15°C day/night.

* Grain could not be analysed. NIL: near iso-genic line.

The first experiment, designated CE HA 1, was set up to repeat an experiment done by Major (1999) on glasshouse grown plants. It would investigate the effect of grain development stage during a three day high temperature treatment (Major, 1999) on PMAA expression in the three CE test varieties Haven, Option and Rialto. Plants were transferred to the 26/20°C day/night high temperature regime when grains of the main tiller were at the late milk and soft dough development stages (section 5.2.2). After treatment, plants were returned to a 20/15°C regime, maintained at a relative humidity of 85%. Plants were kept in CE until all ears of the main tillers had fully senesced. Main tillers were then harvested and thrashed by hand. A skeleton ANOVA for CE HA 1 is given in Table 5.2.

Table 5.2 Skeleton ANOVA for CE HA 1

Source of variation	df
Block	4
Variety	2
Treatment	2
Variety × Treatment	4
Error	32
Total	45-1= 44

During the ear emergence phase of CE HA 1 the electrical supply to the CE cabinet was inadvertently switched off for approximately 24 hours. This caused a fungal infection to take hold in the base of the stems of most plants. In addition to the visible occurrence of disease in the stem bases the infection had also spread to some ears and it probably caused the premature senescence of ears. A score in three categories for the severity of the foot rot present in CA HA 1 was carried out on stem bases only, using Goulds and Polley (1990) as a guide. In addition to the analysis of grains (section 5.2.3), surface-sterilised stem sections were also screened for the presence of the foot rot fungi. Due to the small mass of the stem sections a less rigorous sterilisation protocol was used. As a consequence, the procedure could only be used as a confirmation of the results from the analysis of the grains, as additional fungi isolated could have grown from surviving spores situated on the stem surfaces.

One CE experiment designated CE HA 2 was actually undertaken in a larger CE room at Monsanto Cambridge. Although originally intended to be carried out at Harper Adams, demand for extra CE space due the large number of varieties used, made it necessary for the experiment to be moved to larger facilities. It was designed to test the effect of the high temperature treatment devised by Major (1999) on a broad range of UK winter wheats. To this end eight varieties were grown in CE, chosen for their differences in HFN. During ear emergence however, a serious powdery mildew infection developed, covering a larger part of the leaf surface area. The source of the infection was suspected to be equipment, including coveralls, used in a glasshouse containing a large amount of inoculum. The severity of the infection was however made worse by a design weakness of the experiment. In an attempt to boost numbers, too many plants were packed in a relatively small area, restricting the circulation of air through the foliage. In addition, when powdery mildew took hold fungicide spray could not reach all affected areas. Removal of the plants from

the CE room for a more effective fungicide treatment was not an option due to health and safety restrictions. No substantial amount of grain could be harvested from this experiment, suitable for assessment of α -amylase activity.

CE HA 2 was redone at Harper Adams as CE HA 3, but on a smaller scale using only the three CE test varieties Haven, Option and Rialto. During the milk development stage, plants from CE HA 3 were transferred to a 26/20°C day/night high temperature regime for three days.

Table 5.3 Skeleton ANOVA for CE HA 3

Source of variation	df
Block	3
Variety	2
Treatment	1
Variety \times Treatment	2
Error	15
Total	24-1= 23

However, during vegetative growth plants were insufficiently watered for a period of approximately one week. This could have been a cause for plants developing only main tillers, although a similar phenomenon had been observed in PBI varieties grown in warm glasshouses. After water management was brought back to normal, main tillers were removed in order to stimulate the development of a larger number of tillers. This measure had previously been shown to work for plants grown in warm glasshouses. When the majority of tillers were at the milk development stage, plants were transferred for three days to the high temperature regime. Plants were then returned to a 20/15°C and 85% RH environment, until harvest ripeness. After full senescence of ears (ZGS >92), grain from the five oldest tillers of each plant was harvested and bulked for α -amylase assay.

A skeleton ANOVA for CE HA 3 is given in Table 5.3.

Experiments CE HA 4 and 5 were both lost due to major loss of environmental control. Problems were encountered with temperature and humidity control and the daily regulation of incandescent lighting. CE HA 4 was repeated in a subsequent experiment. No grain was harvested from CE HA 5, set up to test the effect that the duration of a 26/20°C day/night high temperature treatment has on PMAA expression in the three CE test varieties. A second attempt to carry out this experiment also failed, as CE HA 7 was plagued by multiple cabinet breakdowns. A resulting severe infection by *Septoria* quashed any chance of harvesting grain usable for α -amylase analysis.

Experiment CE HA 6 was of a factorial design, examining the effect of a three day 26/20°C day/night high temperature treatment during late milk development of the main ears and the effect of a high humidity of 85% for the duration of the following 20/15°C grain ripening period. When grains in the main ear were at the late milk development stage, plants were temperature-treated. After harvest ripeness and full ear senescence (ZGS >92), grains from the five oldest tillers, including grains from the main stem, were bulk-harvested for each plant, effectively measuring PMAA susceptibility of the whole of the milk development stage, spanning ZGS 71-79. A skeleton ANOVA for CE HA 6 is given in Table 5.4.

Table 5.4 Skeleton ANOVA for CE HA 6

Source of variation	df
Block	2
Variety	2
Treatment	3
Variety \times Treatment	6
Error	22
Total	36-1= 35

On two occasions, starting twelve days before first ear emergence in CE HA 6, the temperature inside the CE cabinet rose to over 30°C causing the over-temperature switch to

shutdown humidity control, air circulation and lighting. This situation persisted for several hours each time, before the temperature was sufficiently lowered so that the cabinet could be restarted. The problem was caused by the ambient temperature in the area housing the CE cabinets reaching approximately 40°C for several hours daily during a four week period. The high temperature treatment and following slow grain drying conditions were applied as planned. No cabinet failures occurred during or after the deliberate high temperature treatment phase. Arrangements were made by the University College to have the area holding the CE cabinets better insulated and have an extractor fan installed, to guard against future high temperature mishaps.

Due to limitations in available CE space, only cv. Rialto was used in experiment CE HA 8. This experiment examined the effect of diurnal temperature differences during a three day high temperature treatment on PMAA formation, during the milk development stage. In the absence of neighbour-effect (effectively an alteration of the micro-climate by neighbouring plots, or plants in this case), plants were transferred to a 26/20°C and 26/10°C day/night high temperature regime when main tillers were at ZGS 74-75. After treatment, plants were returned to a 20/15°C regime, maintained at approximately 85% RH. Plants were kept in a CE until all ears had fully senesced. Main tillers were harvested separately and grain from the four most developed younger tillers was bulked. A skeleton ANOVA for CE HA 8 is given in Table 5.5.

Table 5.5 Skeleton ANOVA for CE HA 8

Source of variation	df
Block	3
Treatment	2
Error	6
Total	12-1= 11

As a result of delays incurred during the project, grain from the final experiment CE HA 9 could not be analysed. This experiment was set up to assess the effect of the Rht-gene on PMAA expression, using the three day high temperature treatment during the milk development stage devised by Major (1999). Six Rht near-isogenic lines with the *rht*, *Rht1*, *Rht2*, *Rht3*, *Rht1+2* and *Rht3+2* alleles were kindly supplied by Dr John Flintham from the John Innes Centre. Grains were harvested and stored.

5.2.5 Statistical analysis

Analysis of variance, without and with adjustment for covariates, and regression analysis, including linear regression in groups, were carried out in Genstat for Windows 6th edition. Data was checked for the nature of the distribution of residuals. Data not normally distributed was transformed to a different scale to better fit a normal distribution, prior to analysis. As most analyses of α -amylase activity improved after Ln-transformation (Evers and Ferguson, 1980) of the data, transformation was used in all analyses of α -amylase activity.

5.3 Results

5.3.1 Disease assessment

Fungi causing foot rot symptoms were found in most stem sections of CE HA 1 plants, confirming that all plants suffered infection to some degree. The same pathogens were also identified in harvested grain samples, raising concerns that the fungal development would interfere with grain α -amylase assessment. However, no IEF isozyme bands were found for any of the samples (Plate 5.3) that did not fit AMY1 and AMY2 bands present in a 72 h

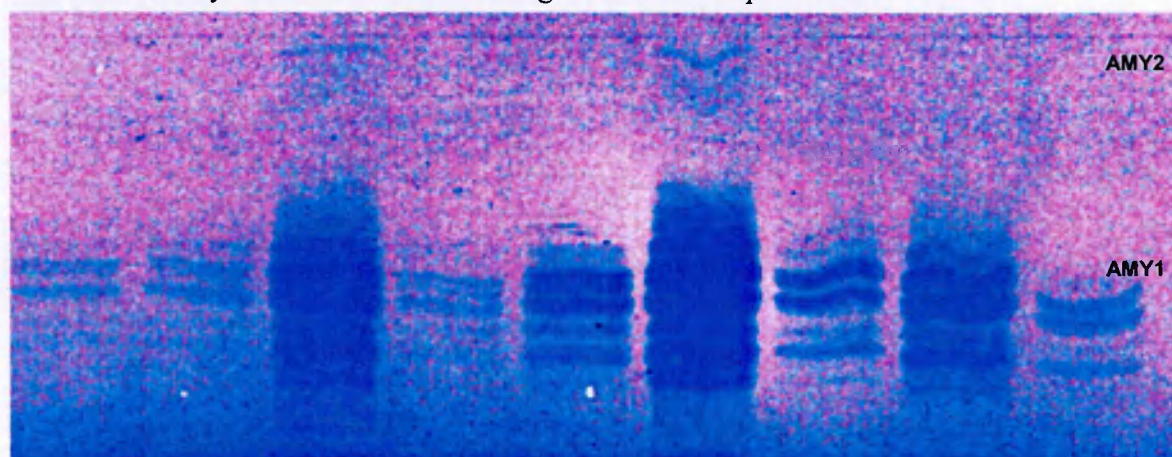
sprouted control sample, of which grains were selected visually for their unsprouted and disease free appearance. This suggests that no significant amounts of α -amylase from non grain sources were present in the samples. Fungal (pI 3.5) and bacterial (pI 5.2) (Alberti *et al.*, 1996) amylase would have appeared as bands above and below the wheat AMY2s. This however does not rule out the possibility of fungal interference with grain α -amylase physiology.

5.3.2 Sprouting assessment

5.3.2.1 Identification of sprouting by visual assessment and FDB and IEF analyses

When grains were harvested for CE HA 1 (see section 5.2.4) it became apparent that small numbers of grain were sprouted. The sprouting is thought to have been induced during a cabinet failure. The presence of elevated hydrolytic enzyme levels was confirmed by IEF (Plate 5.3). As a result a visual assessment of sprouting was made for all subsequent experiments.

Plate 5.3 α -Amylase iso-electric focussing of several samples from CE HA 1



Both AMY1 and AMY2 present in some 30 grain milled samples, with smearing of the AMY1 bands indicating the presence of sprouting. Green filter was used to adjust contrast of negative gel image of α -amylase isozyme electrophoresis (sections 3.1 and 3.2.2). Band positions determined as described in section 3.2.2.

All CE HA experiments except for CE HA 8, which did not suffer from interruptions in environmental control, had some degree of visible sprouting in the grain. A score for visible sprouting is given in Tables 5.7 and 5.8. Due to the nature of the cabinet failure during CE HA 6 the inside cabinet walls and possibly the ears were covered with condensation. For this experiment a visual assessment of sprouting was backed up by additional FDB analyses of samples. The FDB score is given in Table 5.8.

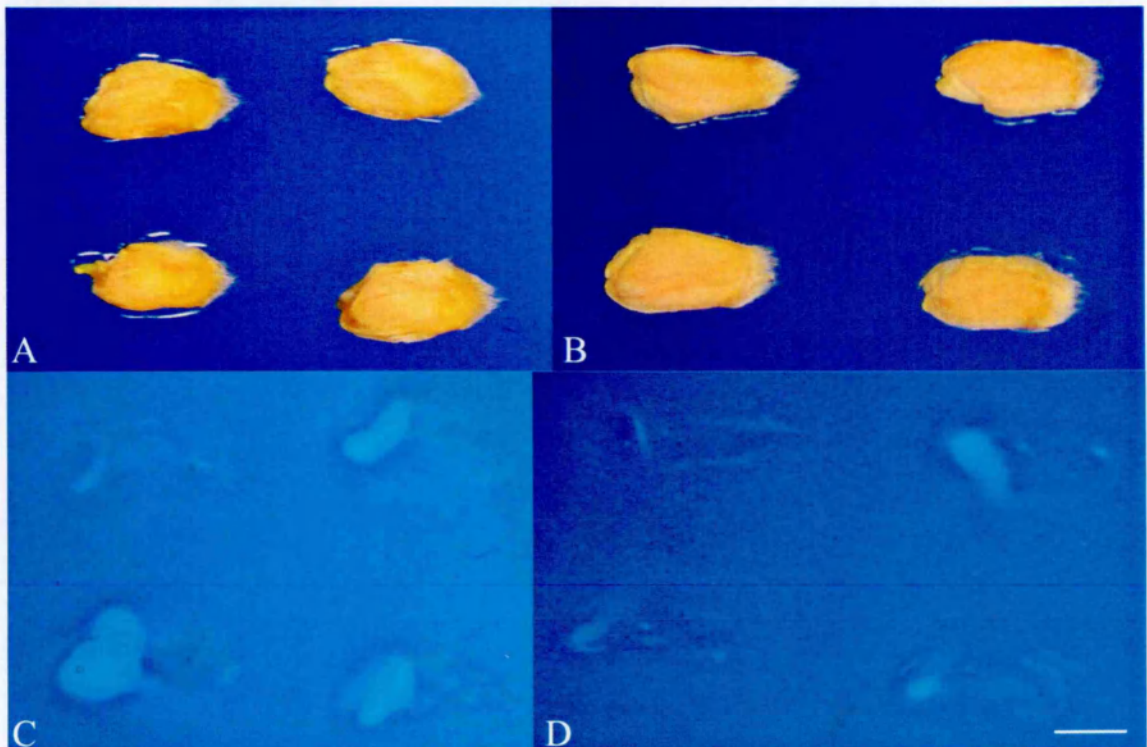
A closer inspection of the equipment showed that condensation formed on the inside walls of the cabinets, mostly near the light boxes, when external temperatures fell below 10°C or when cabinets were temporarily disconnected from the power supply for maintenance during cold weather spells. Better insulation of the area containing the CE cabinets and fewer plants, placed further from the cabinet wall during grain development, could have helped to eliminate sprouting as a problem during the later CE HA 8 experiment.

5.3.2.2 Phadebas gel identification of the location of α -amylase activity in sprouted longitudinal and distal half grains

As well as by means of FDB staining, hydrolytic enzyme activity associated with germination was also observed on Phadebas gel. The Phadebas gel test was however intended to be mainly used for the identification of PMAA (see chapter 3) in experiments. Sprouting, which was not anticipated to be a problem for CE grown material at the start of the project would obscure a clear picture of the amount of PMAA present in the CE experiments. Tests were therefore carried out on sprouted grains from known high HFN varieties, as sound control grains would bring about only minimal digestion of Phadebas gel.

A one hour incubation of longitudinal half grains of 24 h sprouted cv. Malacca and Option (Plate 5.4 A&B) showed clear sprouting-related α -amylase activity in the embryo/scutellar region of the grains (Plate 5.4 C&D). α -Amylase activity observed elsewhere in the grain was however negligible.

Plate 5.4 Longitudinal half grains (top) of 24 h sprouted cv. Malacca (A) and cv. Option (B), and their foot prints (below, Malacca C and Option D) after 1 h incubation on Phadebas gel

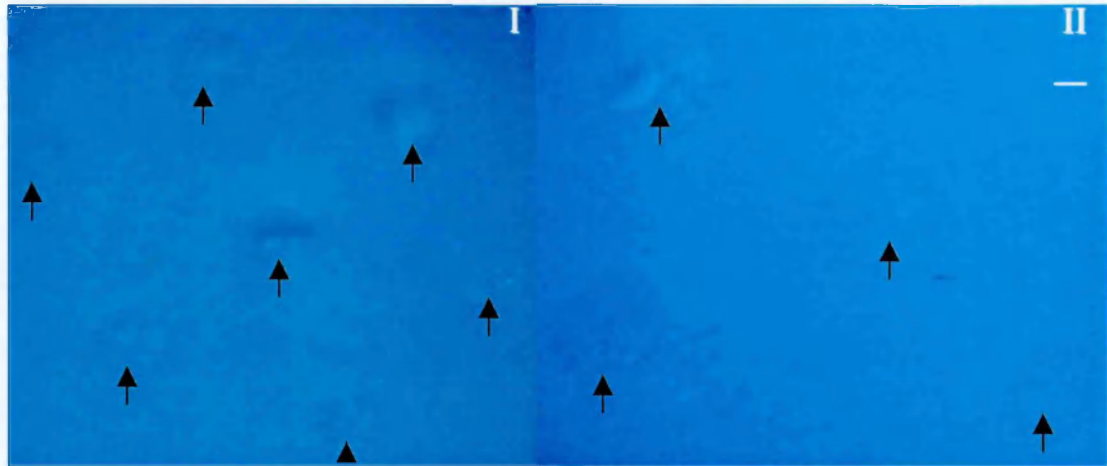


Embryo and scutellum are situated to the left of grains. Eight grains per cv. per Petri dish. Bar corresponds to 3 mm.

Grains sprouted for 24 h, of both cv. Malacca and Option, showed no substantial visible α -amylase activity on Phadebas gel (Plate 5.5) compared to more severely sprouted grain (Plate 5.8), when incubated for 3.5 h on Phadebas gel as distal half grains.

An accompanying visual assessment of the grain showed clear outward signs of embryo activity in some of the 24 h sprouted grains (Plate 5.6) and all of the more severely sprouted grains (Plate 5.9).

Plate 5.5 Footprints of 24 h sprouted grains of cv. Malacca (I) and cv. Option (II) incubated for 3.5 h on Phadebas gel as distal half grains



Transverse cut face of distal half grains (25/cv./Petri dish) placed on gel surface, with crease (see arrows) situated at bottom of grain outline. Bar corresponds to 1 mm.

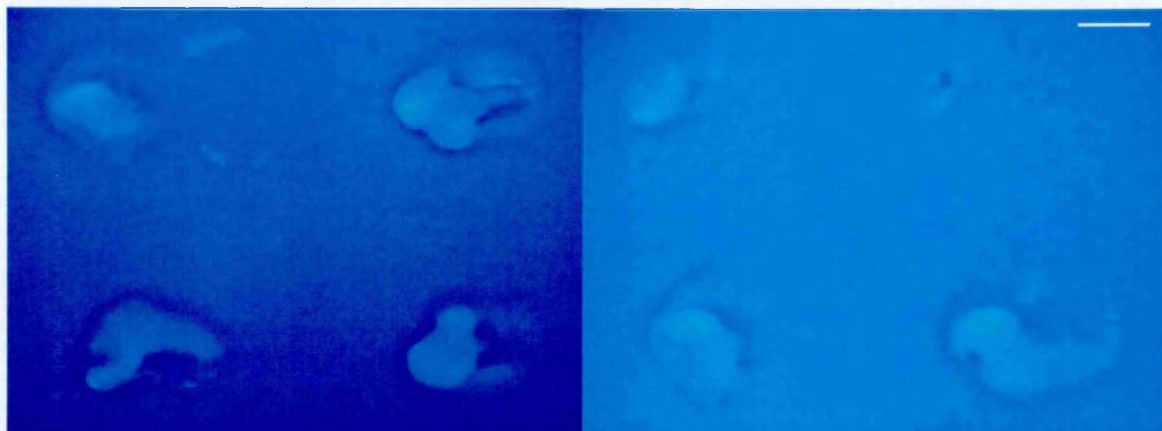
Plate 5.6 Visual assessment of 24 h sprouted grains of cv. Malacca (left) and cv. Option (right)



α -Amylase was detectable throughout the grain in most grains of 72 h sprouted Malacca and Option (Plate 5.7). Although the majority of the α -amylase activity was still concentrated at the embryo end of the grain, in most grains clear activity was also visible away from the embryo/scutellum, as enzyme production by the aleurone layer was initiated.

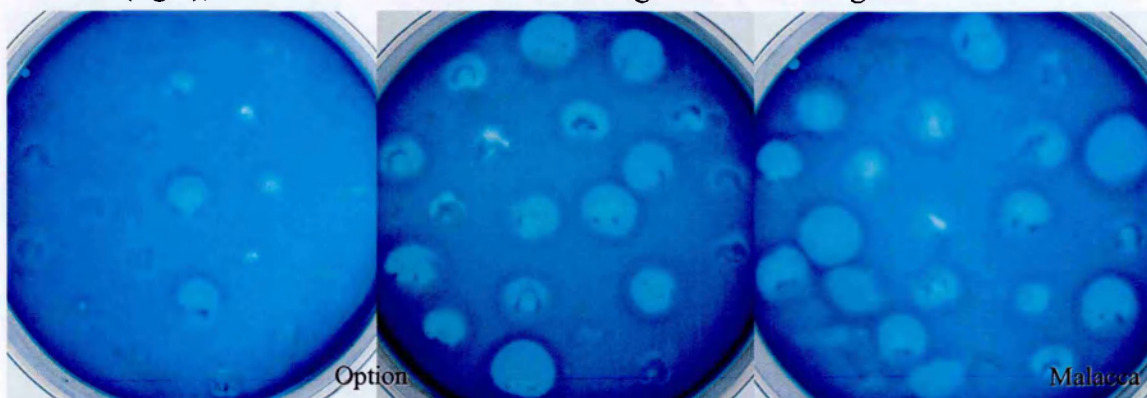
Although just over a third of twenty 48 h sprouted Option grains showed considerable activity as distal half grains incubated for 3.5 h on phadebas gel, almost all of the twenty 72 h Option and 48 h Malacca grains were capable of substantial Phadebas digestion (Plate 5.8).

Plate 5.7 Footprints of 72 h sprouted grains of cv. Malacca (left) and cv. Option (right), incubated for 1 h on Phadebas gel as longitudinal halved grains



Embryo and scutellum are situated to the left of grain outlines. Eight grains per cv. per Petri dish. Bar corresponds to 3 mm.

Plate 5.8 Footprints of 48 and 72 h sprouted grains of cv. Option (left and middle) and cv. Malacca (right), incubated for 3.5 h on Phadebas gel as distal half grains



Transverse cut face of distal half grains placed on gel surface, with crease situated at bottom of grain outline. 20 grains per Petri dish. Petri dishes used in Plates have a 55 mm diameter.

Grains sprouted for 48 and 72 h produced main roots of various lengths (0-12 mm) (Plate 5.9). Most grains had a main root measuring between 5 and 10 mm. Only three out of 50 48 h sprouted grains showed no visible root development, although swelling of the plumule was apparent when the grains were still hydrated.

A similar observation as for cv. Malacca and Option above was made earlier for over two hundred grains of cv. Rialto. Developmental processes in the embryo were clearly evident (Plate 5.10) before considerable levels of α -amylase activity become apparent on Phadebas gel (Plate 4.1).

Plate 5.9 Visual assessment of 48 and 72 h sprouted grains of cv. Option (top and bottom) and 48 h sprouted grains of cv. Malacca (middle)

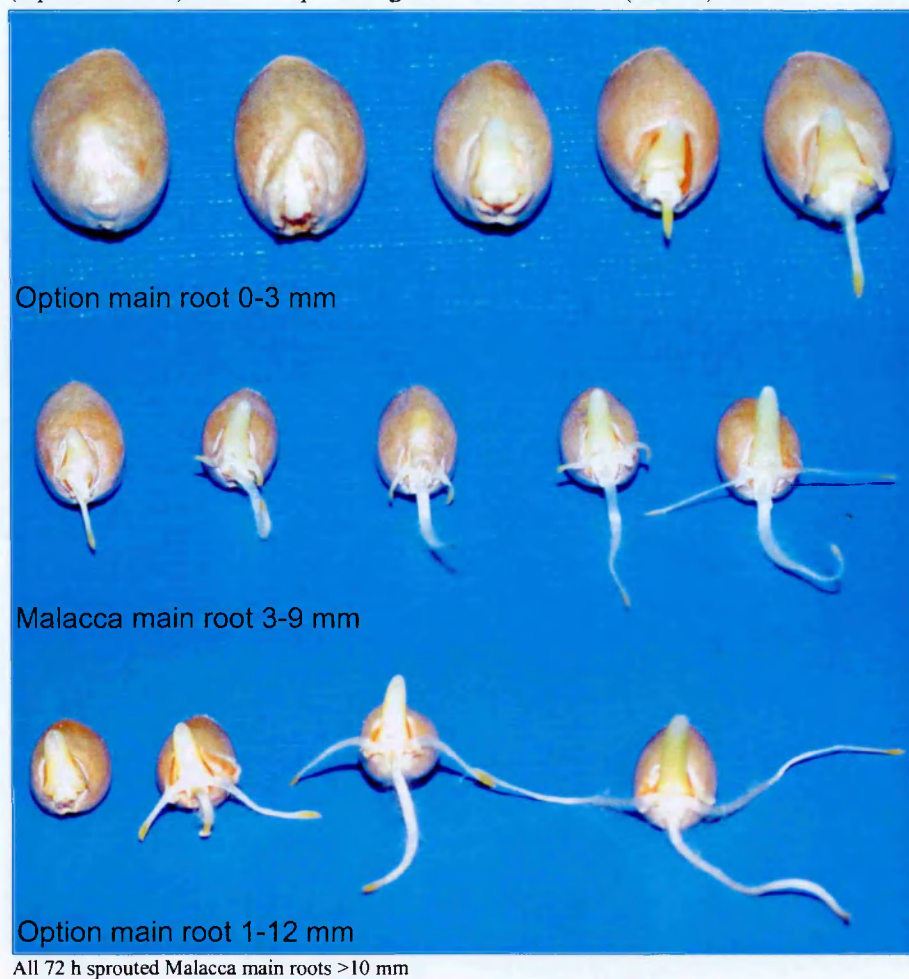
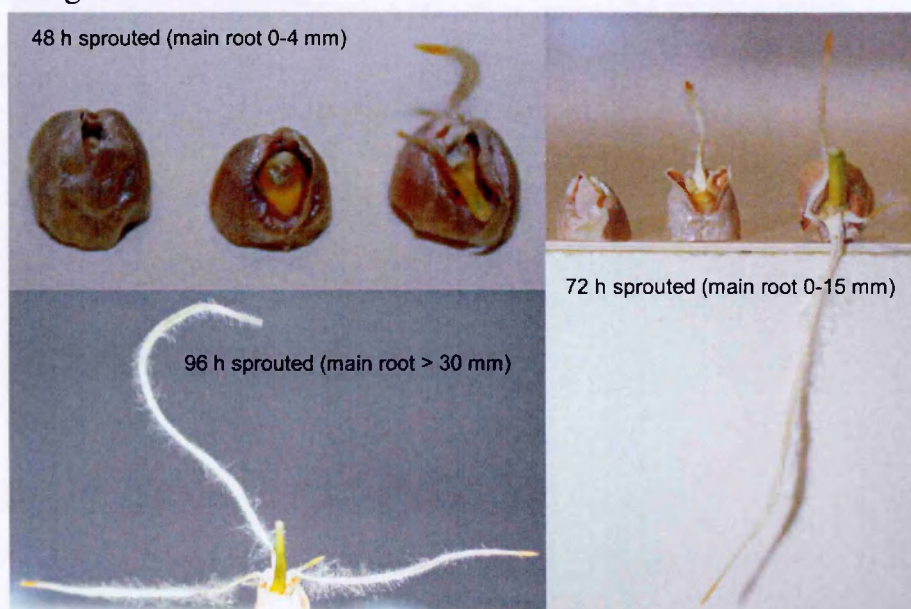


Plate 5.10 Visual assessment of 48 to 96 h sprouted embryo proximal half grains of cv. Rialto



Associated distal half grains (220+ grains ranging 40-100 h sprouting) were incubated on Phadebas gel (see section 4.3.1)

5.3.3 Visual assessment of grain development stage and single grain moisture content at treatment transfer

It has been suggested that grain size affects PMAA levels (Evers *et al.*, 1995). A reduction in the number of grains per ear could result in heavier grains (Abbate *et al.*, 1997) and thus an altered expression of PMAA. As grain development could not be expressed in days after anthesis (section 5.2.2) and as it was felt that the method used by Major (1999) could potentially result in the removal of too many grains from the ears (3 grains for ZGS assessment and freeze-drying), it was important to show that moisture content determination on a single grain per ear (sections 5.2.2 and 5.2.3) correlated well with a visual assessment of grain development.

During the start of the treatment phase for CE HA 6 and 7 a broad range of grain development stages was assessed visually, for a large number of tillers. Data from these two experiments, which covered the milk and dough development stages (section 5.2.2) (Tottman, 1987), was therefore ideal for comparing the visual assessment on three mid-ear grains with single grain moisture content at transfer (Figures 5.2 and 5.3).

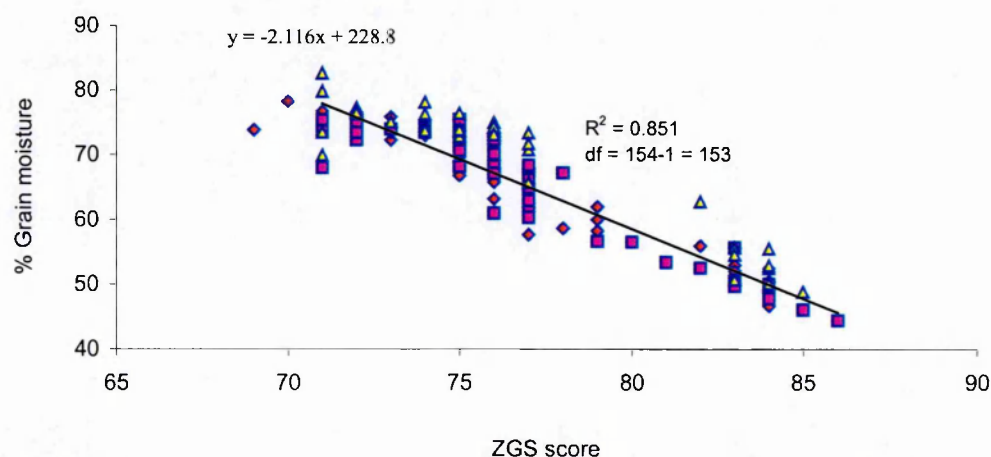


Figure 5.2 Relationship between a visual score for grain development on three mid-ear grains at the beginning of CE HA 6 treatment transfer and moisture content of a single mid-ear grain, with ▲ Haven, ◆ Option and ■ Rialto

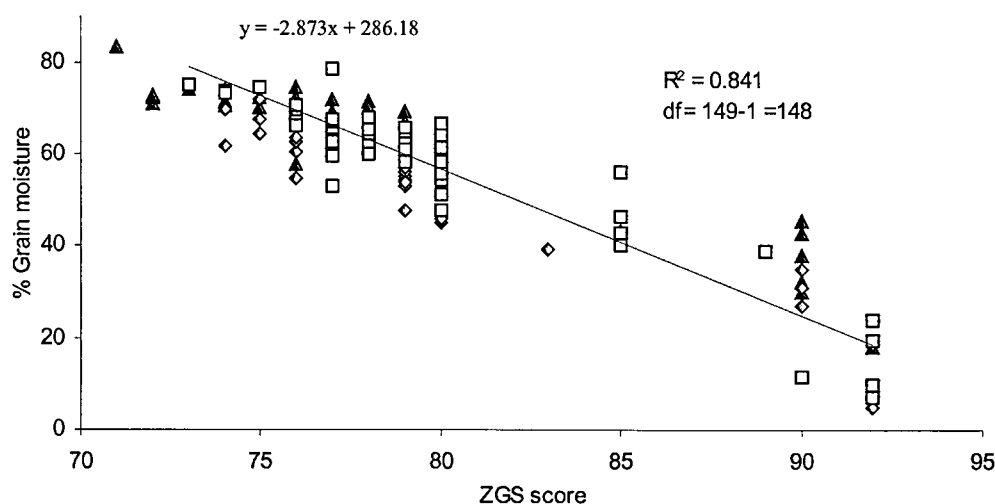


Figure 5.3 Relationship between a visual score for grain development on three mid-ear grains at the beginning of CE HA 7 treatment transfer and moisture content of a single mid-ear grain, with \blacktriangle Haven, \blacklozenge Option and \blacksquare Rialto

The relationship between the visual score for grain development at transfer and single grain moisture content at transfer was largely linear for both experiments, with similar values of 84% (Figure 5.3) and 85% (Figure 5.2) of moisture variance accounted for by ZGS score.

5.3.4 α -Amylase analysis

5.3.4.1 α -Amylase activity in milled grain samples

ANOVA for α -amylase activity of 30 grain milled samples from CE HA 1 (Table 5.6) showed a significant varietal effect. As expected, the average Option α -amylase activity of 275 mEU/g dw was lower than the 446 mEU/g dw average activity for Rialto. Unexpectedly, Haven produced the lowest α -amylase activity of the three varieties, averaging at 273 mEU/g dw. No significant effect of temperature treatment was however found.

Table 5.6 The effect of a high temperature treatment during two grain development stages on α -amylase activity of three varieties used in CE HA 1

		mEU/g	Ln mEU/g
Rialto	Treatment 1	470	6.15
	Treatment 2	403	5.92
	Treatment 3	463	6.14
	Mean	446*	6.07*
Option	Treatment 1	243	5.09
	Treatment 2	208	4.42
	Treatment 3	373	5.57
	Mean	275	5.03
Haven	Treatment 1	223	5.21
	Treatment 2	322	5.58
	Treatment 3	275	5.43
	Mean	273	5.41
	Variety L.S.D.		0.714
	Treatment L.S.D.		0.714
	Treatment \times Variety L.S.D.		1.236
	Variety P value		0.019
	Treatment P value		0.516
	Treatment \times Variety P value		0.594

Treatment 1: no transfer from 20°C/ 15°C regime, Treatment 2:

transfer to 26°C/ 20°C for 3 days at late milk development,

Treatment 3: transfer to 26°C/ 20°C for 3 days at soft dough stage.

*Differs significantly from other treatment means. Samples size is 30 grains.

A score in three categories for the severity of foot rot in stem bases of plants from CE HA 1 related in a significant though weak linear fashion to both α -amylase activity and the natural logarithm of α -amylase activity ($R^2_{\text{activity}} = 0.165$ and $R^2_{\text{Ln activity}} = 0.115$). The subsequent ANOVAs adjusted for the disease covariate, showed no significant varietal, treatment or treatment \times variety interaction with regard to α -amylase activity or the natural logarithm of the activity.

Analysis of 50 grain milled samples from CE HA 3 showed a more familiar pattern of α -amylase activity (section 5.2.1), with Option, Rialto and Haven producing an average α -amylase activity of 36, 220 and 265 mEU/g dw respectively (Table 5.7). Yet only the activity from Option could be shown to be significantly different from other α -amylase activities. While the high temperature treatment (treatment 2) mean α -amylase activity was

higher than the control α -amylase activity, for Option, the reverse was the case for Haven and Rialto. No significant effect of temperature treatment on α -amylase activity was found.

Table 5.7 The effect of a high temperature treatment on α -amylase activity of three varieties used in CE HA 3

		mEU/g	Ln mEU/g	[†] Visible sprouting
Rialto	Treatment 1	261	5.47	2.8
	Treatment 2	179	5.11	0.5
	Mean	220	5.29	-
Option	Treatment 1	33	3.43	0.0
	Treatment 2	38	3.45	0.2
	Mean	36*	3.44*	-
Haven	Treatment 1	281	5.52	1.5
	Treatment 2	249	5.41	0.5
	Mean	265	5.46	-
	Variety L.S.D.	0.643	-	-
	Treatment L.S.D.	0.525	-	-
	Treatment \times Variety L.S.D.	0.909	-	-
	Variety P value	<.001	-	-
	Treatment P value	0.565	-	-
	Treatment \times Variety P value	0.815	-	-

Treatment 1: no transfer from 20°C/ 15°C regime, Treatment 2: transfer to 26°C/ 20°C for 3 days during milk development. *Differs significantly from other treatment means. [†] Number of grains visibly sprouted. Samples size is 50 grains.

A score for visible sprouting displayed only a weak significant linear relationship with α -amylase activity ($R^2 = 0.17$). ANOVA adjusted for the visual score as a covariate again did not improve on the non-adjusted data.

Option harvested from CE HA 6 once more produced significantly lower amounts of α -amylase than Haven and Rialto, averaging at 44 mEU/g dw (Table 5.8). Mean activity of Haven and Rialto was four to five times that of Option. Although not significantly, mean α -amylase activity for Haven was lower than that of Rialto, with 173 and 214 mEU/g dw respectively. No significant effect of temperature treatment or high humidity on α -amylase activity was found.

Table 5.8 The effect of a high temperature treatment and slow grain drying on α -amylase activity of three varieties used in CE HA 6

	Temperature transfer [†]	High humidity after transfer [‡]	mEU/g	Ln mEU/g	▲Visible sprouting	▼FDB	
Rialto	0	0	157	5.00	0.0	0.2	
	1	0	167	5.04	0.6	0.0	
	0	1	306	5.62	0.3	0.0	
	1	1	225	5.27	0.0	0.5	
	mean		214	5.23	-	-	
Option	0	0	38	3.60	0.0	0.2	
	1	0	39	3.65	0.0	0.0	
	0	1	53	3.58	0.0	0.0	
	1	1	45	3.54	0.0	0.0	
	mean		44*	3.59*	-	-	
Haven	0	0	214	5.33	0.0	0.0	
	1	0	142	4.94	0.6	0.6	
	0	1	203	5.11	0.0	0.3	
	1	1	132	4.88	0.3	0.6	
	mean		173	5.07	-	-	
				L.S.D.	P value		
Temperature			0.429	0.469	-	-	
Humidity			0.429	0.734	-	-	
Variety			0.525	<.001	-	-	
Temperature × Humidity			0.606	0.803	-	-	
Temperature × Variety			0.742	0.831	-	-	
Humidity × Variety			0.742	0.489	-	-	
Temperature × Humidity × Variety			1.050	0.863	-	-	

† 0: no transfer to high temperature 1: transferred to 26°C/ 20°C for 3 days at late milk development of main tiller. ‡ 0: no increase in relative humidity 1: high humidity after temperature treatment. *Differs significantly from other treatment means. ▲ Number of grains visibly sprouted. ▼ Number of grains showing FDB fluorescence. Samples size is 50 grains.

Neither a visible sprouting score nor a count of the number of FDB fluorescent grains (Table 5.8) could be used as covariates in the analysis of α -amylase activity as neither of the two sprouting scores correlated well with the α -amylase measurements ($r_{\text{vis. spr.}} < 0.12$ and $r_{\text{FDB}} < 0.098$). In addition, regression analysis revealed no significant linear relationship between the two sprouting scores and either α -amylase activity or the natural logarithm of α -amylase activity.

Analysis of grain from CE HA 8 Rialto exposed a much higher α -amylase activity on average in bulked grain from younger tillers than was present in grain from main tillers, with the activity of younger tillers more than double that of main ears. Although not

significant, average α -amylase activities of both treatment 1 (control) and treatment 3 (large diurnal temperature difference) were higher than the activity of treatment 2 (the 26/10°C temperature treatment), in older tillers (Table 5.9). The reverse was the case in younger tillers, with the 26/10°C temperature treatment, although not significant, producing higher activities than either control or large diurnal temperature treatment.

Table 5.9 The effect of two high temperature treatments on α -amylase activity in grain from the main stem and four primary tillers of cv. Rialto in CE HA 8

	Main stem Ln mEU/g (mEU/g)	Four primary tillers Ln mEU/g (mEU/g)	Difference [†] of Ln-transformed
Treatment 1	4.20 (67.5)	4.82 (124)	0.62
Treatment 2	3.95 (52.8)	5.28 (203)	1.33*
Treatment 3	4.11 (61.5)	4.96 (154)	0.85
L.S.D.	0.363	0.533	0.471
P value	0.314	0.170	0.026

Treatment 1: no transfer from 20°C/ 15°C regime, Treatment 2: transfer to 26°C/ 20°C for 3 days during milk development main tillers, Treatment 3: transfer to 26°C/ 10°C for 3 days during milk development main tillers. [†] Four tiller value minus main tiller value. *Differs significantly from other treatments.

Sample size main tiller is 30 grains, sample size bulked tillers is 120 grains.

To compare the effect of temperature on older grains with the effect of temperature on younger grains, an ANOVA was carried out on the difference between α -amylase activity of the two. This was done because measurements on the different tillers of one plant are not independent of each other and can therefore not legitimately be used as separate factors in an analysis of variance.

When the data for older and younger tillers was combined (right-hand column, Table 5.9), the value of treatment 2 was significantly higher than the other treatments. However, although not significantly, all CE experiments showed the potential of a high temperature period to reduce α -amylase activity.

5.3.4.2 Phadebas gel analysis of grain sub-samples with no visible sprouting

As it is possible that sprouting (see Tables 5.7 and 5.8 for sprouting score) had a confounding effect on measured α -amylase activity as an indicator of the difference in PMAA expression between treatments, follow up tests on Phadebas gel were carried out with grain samples re-assessed for visible signs of sprouting. Visibly sprouted grains were removed from the samples.

The Phadebas gel test could not be done for grains from CE HA 1 as the majority of the harvested grain was lost during a high temperature and high humidity period, when environmental control was lost in the grain storage area. Sub-samples from surviving grain did show the presence of PMAA to some degree in all three CE test varieties.

The amount of grain available after α -amylase analysis of CE HA 3 was not sufficient for an additional Phadebas gel test.

Not all samples from the factorial experiment CE HA 6 looking at the effect of temperature and humidity on PMAA formation yielded enough grain for a full additional Phadebas analysis. Six missing values for Haven and one for Rialto were estimated in Genstat 6th edition.

Although Option produced almost no Phadebas discoloration (2 grain mean), on average more than half of the Haven (33.99 grains) and Rialto grains (27.34 grains) were capable of noticeable Phadebas digestion, mainly apparent in the crease region (figures are presented in Table 5.10). While no significant effect of temperature treatment was found on the number of grains active on Phadebas gel, variety, humidity, and the interaction,

Table 5.10 Mean numbers of grains showing α -amylase activity on Phadebas gel in 50 grain samples from CE HA 6

	Temperature transfer†	High humidity after transfer‡	Number of grains active on Phadebas gel	
Rialto	0	0	28.00	
	1	0	36.00	
	0	1	26.05	
	1	1	19.33	
	mean		27.34	
Option	0	0	1.7	
	1	0	4.3	
	0	1	1.3	
	1	1	0.7	
	mean		2.0	
Haven	0	0	32.94	
	1	0	41.55	
	0	1	32.55	
	1	1	28.94	
	mean		33.99	
High temperature mean			15.08	
Low temperature mean			14.26	
High humidity mean			11.85	
Low humidity mean			17.50	
			L.S.D.	P value
Temperature			2.595	0.274
Humidity			2.595	<.001
Variety			3.178	<.001
Temperature × Humidity			3.669	<.001
Temperature × Variety			4.494	0.806
Humidity × Variety			4.494	0.077
Temperature × Humidity × Variety			6.355	0.168

[†] 0: no transfer to high temperature 1: transferred to 26°C/ 20°C for 3 days at late milk development of main tiller. [‡] 0: no increase in relative humidity from 70% 1: 85% high humidity after temperature treatment. Missing values estimated in Genstat 6th edition.

proved to be highly significant. The control treatment, which does not promote slow grain drying, and the slow grain drying treatment seem to produce similar activity on Phadebas gel. However, combining the high temperature treatment with slow grain drying significantly reduces enzyme activity. It is the high temperature treatment on its own that allows for large amounts of enzyme to be accumulated in apparently all three varieties, although at far lower levels in Option.

As with α -amylase activity of CE HA 8 Rialto in the previous section, more suspected PMAA was found in grains of treatment 1 (20/15°C regime) and treatment 3 (26/10°C)

applied to the older main tillers (Table 5.11). However treatment 2 (26/20°C) produced a higher mean number of grains capable of Phadebas digestion in the younger grains. None of the figures within one tiller type were however significantly different from each other.

Table 5.11 Mean numbers of Rialto grain from CE HA 8 showing α -amylase activity on Phadebas gel

	Main stem [▲] (%)	Four primary tillers [▼] (%)
Treatment 1	4.50 (18)	18.2 (36.4)
Treatment 2	3.50 (14)	21.0 (42.0)
Treatment 3	5.75 (23)	18.8 (37.6)
L.S.D.	4.383	13.27
P value	0.495	0.867

Treatment 1: no transfer from 20°C/ 15°C regime, Treatment 2: transfer to 26°C/ 20°C for 3 days during milk development main tillers, Treatment 3: transfer to 26°C/ 10°C for 3 days during milk development main tillers. ▲ 25 grain sample. ▼ 50 grain sample.

5.3.5 α -Amylase in relation to development stage at temperature treatment and post-harvest grain characteristics

Major (1999) reported the milk grain development stage to be more susceptible to PMAA induction than the dough development stage. As results from CE HA 8 (Table 5.11) suggest that at least for Rialto the susceptible period might be even earlier, α -amylase activity was examined in relation to measurements of grain development stage. In addition, α -amylase activity has been linked to grain size (Evers *et al.*, 1995), with larger grains more inclined to produce greater amounts of enzyme. The relationship between grain α -amylase and grain dry mass was therefore also scrutinised.

Since temperature treatment did not show a significant effect on either α -amylase activity or the number of grains active (Tables 5.6, 5.7, 5.8 and left and middle columns of 5.9) on Phadebas gel (Tables 5.10 and 5.11), it was felt that data from the control and high temperature treatment could be combined to give an indication as to the relationship between α -amylase and development stage or additional post-harvest measurements.

No sizeable correlation ($r_{\text{amylase activity}} = -0.071$, $r_{\text{Ln amylase activity}} = -0.087$) nor any significant linear relationship, either for the combined data or single varieties, was found between a ZGS score (ZGSs and range of grain moisture content, for Haven, Option and Rialto, shown in Figure 5.2) and (natural logarithm of) enzyme activity for CE HA 6. No significant linear relationship was found for grain moisture content at treatment transfer and enzyme activity for the different varieties, but a weak relationship ($r = 0.159$) did present itself for all of the data ($R^2 = 0.019$, $P < 0.001$). This linear relationship was slightly improved by converting amylase activity to a natural logarithm scale ($R^2 = 0.051$, $P < 0.001$). However, as the regression lines are almost horizontal, it can be argued that no real relationship exists between the two parameters.

Linear regression analysis in groups again revealed a poor relationship between α -amylase activity or Ln- α -amylase activity, and grain development stage at treatment transfer, when temperature treatment and humidity treatment were examined separately (See previous section, ANOVA showed no significance of either temperature or humidity treatment in general). When the same relationship was examined, but grouped for the three varieties, a significant linear relationship was found for cv. Haven and Rialto (Figure 5.4), but with a non-significant near horizontal gradient. Thus, there is effectively no difference between the α -amylase activities of grains receiving treatment during earlier and later grain development stages.

A similar regression analysis in groups with results from additional Phadebas tests yielded more interesting results. The number of grains showing α -amylase activity on Phadebas gel was viewed in relation to the average ZGS score for a bulked grain sample. Although regression analysis in groups found no significant linear relationship for Option, significant

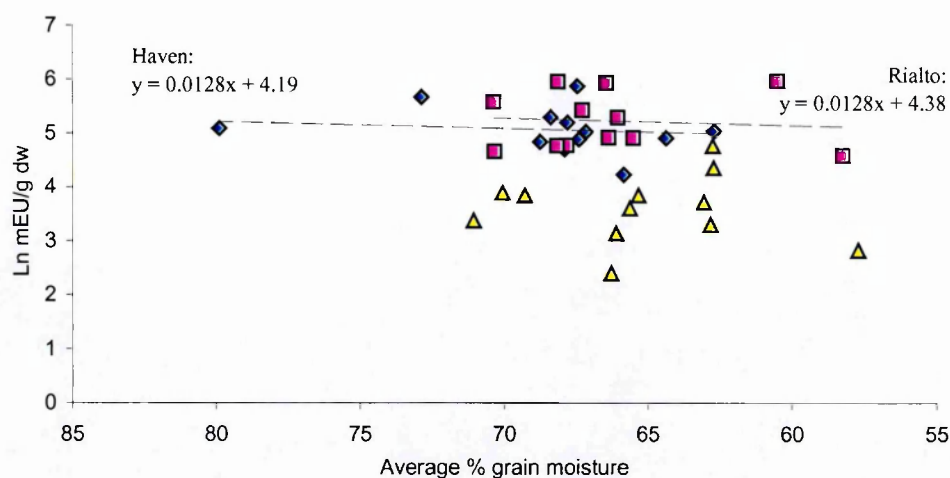


Figure 5.4 Relationship between average grain moisture content at the time of high temperature treatment transfer CE HA 6 and the natural logarithm of sample α -amylase activity of harvested grain, with \blacklozenge Haven, \blacktriangle Option and \blacksquare Rialto

but opposite slopes were found for Haven and Rialto (Figure 5.5). For cv. Haven this means that the number of α -amylase active grains increased when high temperature transfer was carried out later in grain development. The reverse, however, was found for cv. Rialto, with more grains becoming α -amylase active when temperature transfer happened earlier during grain development.

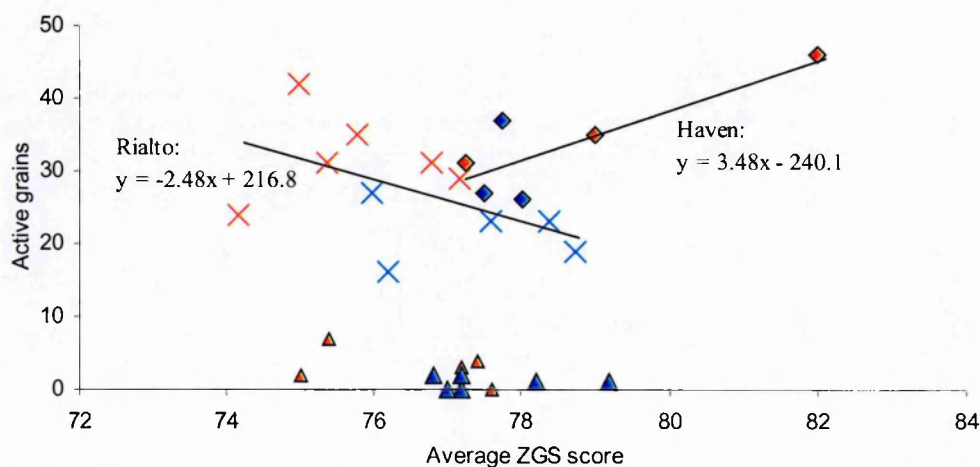


Figure 5.5 Relationship between average ZGS at treatment transfer CE HA 6 and the number of grains showing α -amylase activity on Phadebas gel, with \blacklozenge Haven, \blacktriangle Option and \times Rialto, low humidity applied to red symbols and high humidity to blue symbols

There is however a difference between average grain development stage at time of transfer of high humidity and low humidity treatments (sections 5.2.2 and 5.2.4), as there is a lack of overlap is apparent (Table 5.5). No significant relationship was found when the data from Figure 5.5 was regressed for low and high humidity treatment separately.

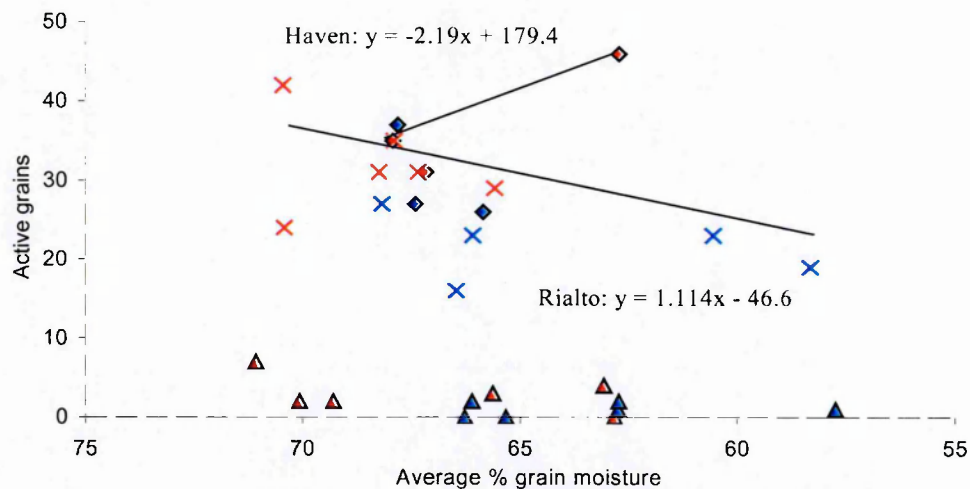


Figure 5.6 Relationship between average grain moisture content at treatment transfer CE HA 6 and the number of grains showing α -amylase activity on Phadebas gel, with \diamond Haven, \triangle Option and \times Rialto, low humidity applied to red symbols and high humidity to blue symbols

The second measure for grain development, the percentage grain moisture at treatment transfer, was subsequently also looked at in relation to Phadebas gel activity (Figure 5.6).

The results mirror those found for the relationship between average ZGS at time of treatment and the number of grains showing enzyme activity on Phadebas gel. A linear relationship exists between ZGS and grain moisture content (Figures 5.2 and 5.3).

As no significant effect of treatment was found on α -amylase activity, all dry mass data was regressed against enzyme activity (Figure 5.7 and 5.8). The amylase activity was ln-transformed to make it more normally distributed, and better presentable in a graph. The combined CE HA 3 data showed no linear relationship to exist between post-harvest grain dry weight and grain α -amylase activity ($r = 0.338$). Regression analysis with varieties as

groups showed a positive trend for the varieties Haven and Rialto, although regression for the two separately failed to show any significant relationship. On the other hand, cv. Option displayed a significant negative linear relationship ($R^2 = 0.831$ ($P = 0.001$)) even when analysed separately. All three slopes were however $< |0.05|$. Therefore, within the dry matter range observed, Ln α -amylase remains near constant.

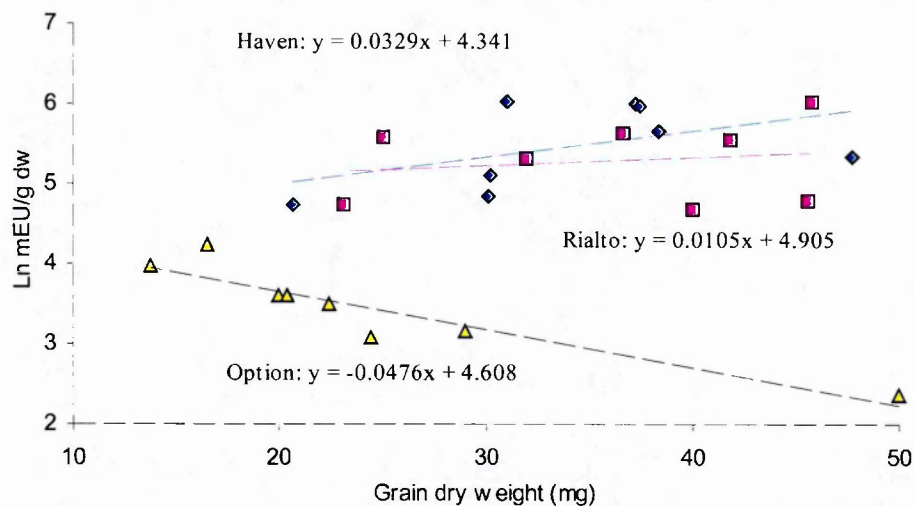


Figure 5.7 Relationship between average post-harvest grain dry weight and the natural logarithm of α -amylase activity in grain samples bulked for the five most developed tillers of CE HA 3, with \blacklozenge Haven, \blacktriangle Option and \blacksquare Rialto

The combined CE HA 6 data showed a weak positive trend towards an increase in enzyme activity as grain weight increased ($R^2_{\text{Ln amylase activity}} = 0.112$ ($P = 0.026$)) (Figure 5.8).

Regression analysis in groups showed no significant contribution of 'dry mass \cdot variety' to the model. Re-analysis of the data for the reduced model with one gradient for the three varieties exposed significant positive linear relationships for Haven, Option and Rialto. Interestingly, while a positive gradient was found for the combined data, regression analysis in groups suggested the relationship within varieties to be of a negative nature, with no significant difference in slope between the varieties. Thus, while α -amylase activity decreased with increasing grain size within a variety, α -amylase activity tended to be higher in the varieties with larger grain size. However, as a maximum increase in

enzyme activity of only 5 to 6 mEU/g dw can be expected over the full range of measured grain dry mass, the latter does not seem to noticeably influence the amount of α -amylase produced. These results are similar to those found for CE HA 3.

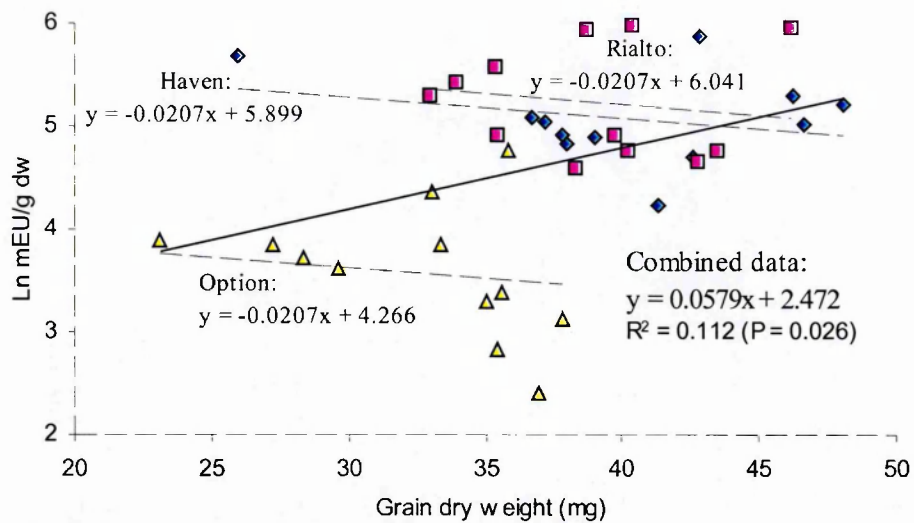


Figure 5.8 Relationship between average grain dry weight and the natural logarithm of α -amylase activity in grain samples bulked for the five most developed tillers of CE HA 6, with ♦ Haven, ▲ Option and ■ Rialto

The same type of analysis was carried out for the data derived from additional Phadebas gel tests with grain from CE HA 6. Regression analysis in groups for Haven, Option and Rialto, could not detect a significant linear relationship for cv. Option, between average grain dry weight and the number of grains active on Phadebas gel. While a significant relationship was found for Haven and Rialto, the near horizontal slopes were not significant. It needs to be noted at this point that the number of grains with PMAA in a sample is just one factor that influences the strength of PMAA in the sample and that for example variation in single grain PMAA activity would also influence the overall PMAA activity.

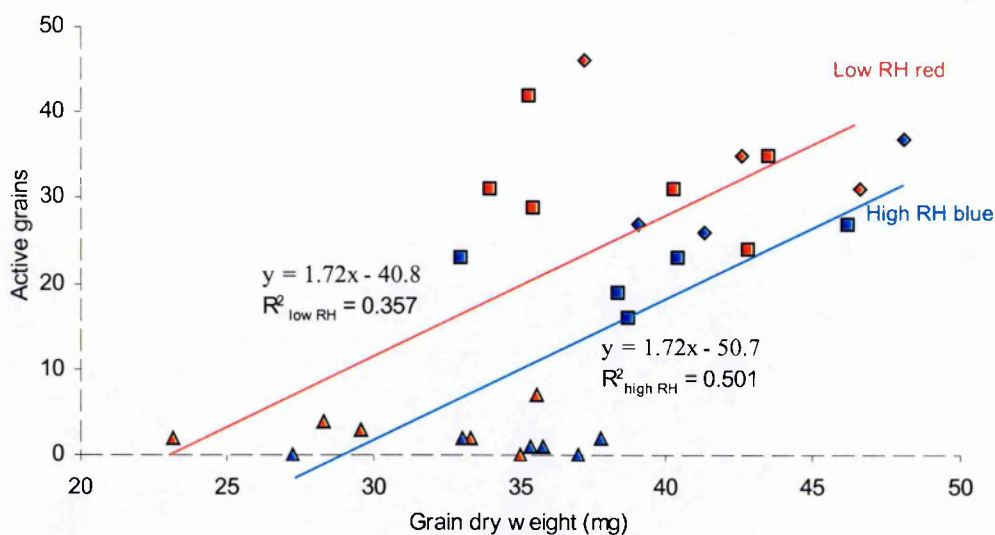


Figure 5.9 Relationship between average grain dry weight of samples bulked for the five most developed tillers of CE HA 6 and the number of grains showing α -amylase activity on Phadebas gel, with \diamond Haven, \blacktriangle Option and \blacksquare Rialto

Figure 5.9 shows the relationship between post-harvest grain dry weight and the number of grain showing α -amylase activity on Phadebas gel, but grouped as low and high humidity samples. The red regression line is associated with low humidity treatment and the blue regression line with high humidity. When individual varieties are disregarded, regression analysis in groups reveals an average increase of almost 6 mg dry mass in samples from plants grown in a humid environment from early grain development onwards. A similar assessment of the data, but grouped for the control temperature and high temperature treatment did not expose any significant relationships.

For CE HA 8, both sample α -amylase activity and the number of grains showing activity on Phadebas gel correlated poorly with grain moisture content at treatment transfer. While enzyme activity showed some positive correlation ($r = 0.27$), Phadebas data exhibited a negative trend ($r = -0.28$). However, plants were transferred to a high temperature treatment within a narrower range of main ear grain development (ZGS 74-75), compared to other experiments.

The possibility to investigate the relationship between the number of grains per ear and α -amylase parameters (see section 2.4.2) for CE HA 8 was limited by the fact that the two groups of ears, main tillers and bulked younger tillers, are not independent. Thus, not meriting either an ANOVA (adjusted for covariates) for the complete set of data, or linear regression analysis in groups. However, a crude analysis providing an indication as to the nature of the relationship between the number of grains in an ear and enzyme measurements was made for the full set of data from CE HA 8. Earlier ANOVAs had not revealed any significant temperature treatment effect on either α -amylase activity (Table 5.9) or the number of grains active on Phadebas gel (Table 5.11). This further basic examination did bring to light a weak linear relationship between the number of grains in an ear and grain α -amylase (Figure 5.10). While no significant linear relationship was found for the main ears ($r_{\text{Phadebas}} = -0.31$, $r_{\text{Ln-}\alpha\text{-amylase}} = -0.065$) and bulked younger tillers ($r_{\text{Phadebas}} = -0.5$, $r_{\text{Ln-}\alpha\text{-amylase}} = -0.29$) separately, a suggestion of a trend through the complete set of data was given by the presence of a weak linear relationship between the grain count and Ln- α -amylase ($R^2_{\text{Phadebas}} = 0.338$, $R^2_{\text{Ln-}\alpha\text{-amylase}} = 0.282$). Post-harvest grain α -amylase activity therefore seems to decrease in both older and younger tillers, as the number of grains per ear gets larger.

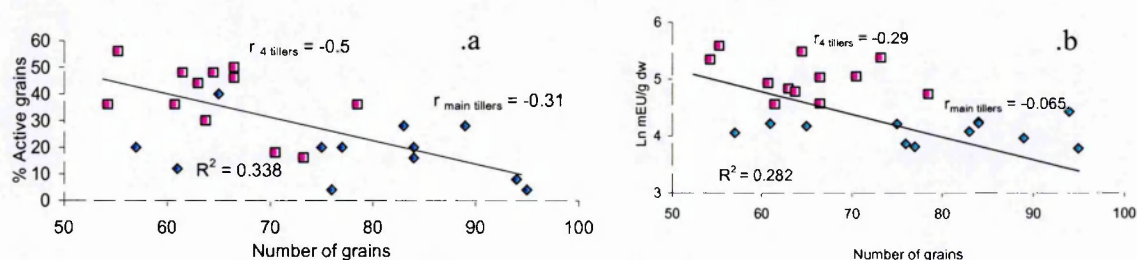


Figure 5.10 Relationship between the ♦ number of grains per main tiller and the ■ average number of grains per ear in four younger tillers of CE HA 8, and (a) percentage of grains showing α -amylase activity on Phadebas gel and (b) sample Ln- α -amylase activity, with regression lines calculated for data from main and younger tillers combined

A similar look at the relationship between average post-harvest grain dry mass and α -amylase parameters (Figure 5.11) also revealed a suggestion of a trend for the combined data ($R^2_{\text{Phadebas}} = 0.248$, $R^2_{\text{Ln-}\alpha\text{-amylase}} = 0.333$). However, no significant linear relationship was found for the main ears ($r_{\text{Phadebas}} = 0.24$, $r_{\text{Ln-}\alpha\text{-amylase}} = 0.047$) and bulked younger tillers ($r_{\text{Phadebas}} = -0.32$, $r_{\text{Ln-}\alpha\text{-amylase}} = 0.22$) separately. While there is a negative trend between grain dry mass and the two enzyme measurements when data of the older and younger tillers is combined, three out of the four correlation coefficients for tiller age group are positive. Interpretation of the data presented in Figure 5.11 is hence less straightforward compared to data presented in Figure 5.10. Although the majority of correlations are positive, thus hinting towards an increase in the number of α -amylase active grains when average grain mass increases, grains from the main tillers appear to produce less α -amylase at higher dry matter contents, compared to younger tillers.

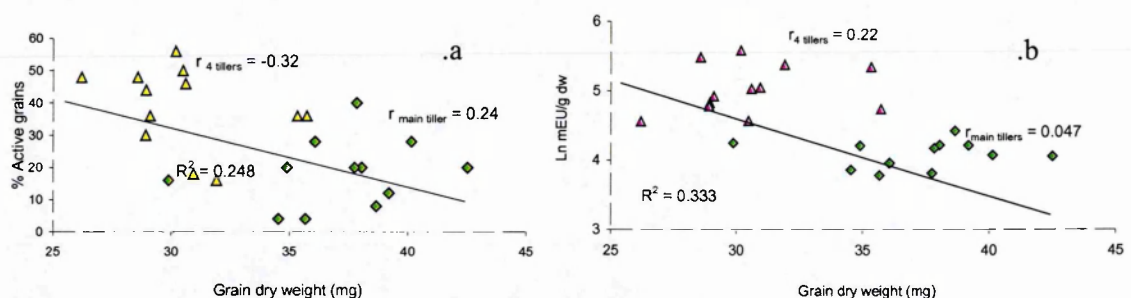


Figure 5.11 Relationship between average grain dry weight of the \blacklozenge main ear and \blacktriangle four younger tillers from CE HA 8 and (a) percentage of grains showing α -amylase activity on Phadebas gel and (b) sample Ln- α -amylase activity, with regression lines calculated for data from main and younger tillers combined

5.4 Discussion

Although, as expected, there was a clear varietal effect on post-harvest grain α -amylase activity (Tables 5.6, 5.7 and 5.8), no significant effect of temperature was found (Tables 5.6, 5.7, 5.8 and left and middle columns 5.9), except when data from younger and older tillers (CE HA 8) was combined (right-hand column Table 5.9). This suggests that a

transient high temperature period induces grain α -amylase formation during earlier grain development stages than examined thus far. While a transient 26/20°C temperature regime induced increased α -amylase formation during these earlier grain development stages, compared to the more mature grains in the milk development bracket, interestingly, a larger (26/10°C) diurnal temperature difference did not induced an increase in α -amylase formation (Table 5.9).

Levels of grain α -amylase were also significantly affected by post-temperature-treatment relative humidity. However, the significant effect of humidity treatment on the number of grains active on Phadebas gel (Table 5.10) could be partly due to a difference in average grain development stage at the time of treatment. When the position of the red symbols is compared to the blue in Figures 5.5 and 5.6, there does not appear to be a full overlap within a variety.

Interestingly, analysis of the CE HA 6, 2×2 factorial (temperature treatment and humidity) data revealed that not a high temperature treatment followed by slow grain drying, but rather a high temperature treatment alone, not accompanied by a subsequent rise in humidity, results in an increase in the number of grains producing α -amylase activity in the crease region. This is in contrast with findings by Gold and Duffus (1996) and Major (1999), that a high temperature treatment followed by slow grain drying can promote PMAA formation. Moreover, the results from experiment CE HA 6 imply that the combination of high temperature treatment followed by high humidity lowers the number of grain with suspected PMAA, compared to a control group or a high humidity treatment during grain filling alone.

An analogous apparent discrepancy between studies has been reported by Armstrong *et al.* (1982). In laboratory experiments with immature grain, King and Gale (1979) and Nicholls (1979) reported sensitisation of detached grains to GA under conditions that allowed for no appreciable desiccation, namely 100% RH. This seemingly contradicts a report by Evans *et al.* (1975) that immature barley grains do not respond to GA unless they are first subjected to a period of dehydration. Armstrong *et al.* (1982) proposed that even in a 100% RH environment, drying or re-distribution of water occurs in detached wheat grain and that there is a window in grain moisture content in which GA-sensitisation takes place. King and Gale (1979) and Nicholls (1979) also reported that slow grain drying results in more enzyme production in detached grains compared to rapidly dried grain. Armstrong *et al.* (1982) however, did not replicate this effect in their experiments. Conversely, in a later paper, Nicholls (1986) rejected the claim by Armstrong *et al.* (1982) that rather than a detachment of the grains from the ear, a period of enforced drying is required for GA-sensitisation. Nicholls (1986) regarded the suggestion of water re-distribution in the grain under 100% RH conditions as highly unlikely. Nicholls (1986) did however moderate the previous conclusion (Nicholls, 1979) and suggested that given the appropriate grain growth conditions detachment of grains from ears is sufficient and dehydration is not necessary for the development of GA sensitivity.

When all these findings are considered, it could well be that it is not the presence or lack of drying as such, but rather the prevention of a rapid transgression of a GA-sensitive window in grain moisture content, that allows for a longer and/or greater accumulation of α -amylase in the grain, given that the pre-treatment grain history allows this. In CE HA 6, the high humidity following the temperature treatment could have kept grain moisture content above the *in vivo* range in which aleurone develops sensitivity to GA, or produces pre-mature α -amylase, for longer. The finding by Nicholls (1979) that aleurone

sensitisation can take place in near 100% RH applies to detached grains, for which Armstrong *et al.* (1982) have suggested a within-grain drying mechanism possibly based on water re-distribution. In contrast, immature grains *in vivo* are connected to a steady supply of moisture from the plant. The combination of high temperature and high humidity could have shortened the period during which aleurone cells were actively producing α -amylase, compared to the grains that were only exposed to high humidity during development. The high temperature-treated slow-drying grains would, at the same moment in time, have been at a different grain development stage than grains that did not receive a high temperature treatment, but were exposed to high humidity during development. Mrva and Mares (2001a) did not report the necessity for a higher relative humidity during grain development when they successfully induced LMA formation in spring wheat grown in Australia. It is not clear from the literature whether drying brings the grain within a moisture range where environmental factors induce aleurone sensitivity to GA, or if the rate of drying itself can induce a reaction from the aleurone in the form of α -amylase secretion. What is interesting to note, is that using scanning NMR, Armstrong *et al.* (1982) showed that grain drying takes place from the dorsal part of the grain towards the crease, indicating a possible water transfer in this direction.

Sensitivity to GA is also modified by temperature (Norman *et al.*, 1982). Results from experiments CE HA 6 and 8 suggest that a transient elevation in temperature during grain development can affect α -amylase formation in the grain. According to Norman *et al.* (1982) temperature treatments can replace dehydration in transforming aleurone tissues from a non-GA-sensitive state to a sensitive state. They reported that a high temperature treatment ($> 27^{\circ}\text{C}$) followed by rapid cooling to a certain temperature, effectively a temperature shock, was required for elevated α -amylase formation by the aleurone of de-embryonated immature grains of wheat cv. Sappo. They found that prolonged incubation

of de-embryonated grains below 27°C did not effect a change in sensitivity. Work by Major (1999) and results from experiment CE HA 8 with cv. Rialto suggest grains in early development to be more susceptible to a high temperature shock than older grains. Major (1999) found that a transient three day shift from a cool to high temperature regime, rather than the reverse, a high to cool temperature and back, was effective in inducing PMAA *in vivo*. This could suggest that a temperature shock is perceived as such, when the temperature difference is greatest relative to the temperature history of the grain.

Grain development in UK winter wheat occurs in relatively cool temperatures. The Australian equivalent of PMAA in spring wheat, LMA, was found to be most effectively induced by a cool temperature treatment (Mrva and Mares, 2001a). Grain development of spring wheat grown in Australia transpires under much higher temperature conditions than found in the UK. The requirement for a short transient temperature shock is however in apparent discordance with other studies, that failed to recognise this requirement prior to induction of elevated α -amylase secretion by the aleurone. Nicholls (1982) reported on the effect temperature has on GA-sensitisation, but did not find a necessity for a temperature shock. As the latter needs to be short-lived according to Norman *et al.* (1982) to have an effect, a high temperature treatment was applied in experiment CE HA 8 with a larger difference in day/night temperature than the high temperature treatment described by Major (1999). It was reasoned that the large and swift shifts in air temperature would be perceived by the grain as a Norman *et al.* (1982)-like shock *in vivo*. This hypothesis was backed by measurements taken in the field during grain development of a number of Rialto crops producing PMAA (chapter 4) and had been proposed earlier by Gold (1991). The Rialto field data revealed that large increases in maximum daily temperature tended not to be matched by the accompanying minimum daily temperatures. The large diurnal temperature treatment applied in CE HA 8 did not however produce any significant

difference in either α -amylase activity or number of grains producing α -amylase in the crease, compared to a control treatment. One possible explanation could be that it is not the large difference in diurnal temperature that is perceived as a shock, but rather the rate of temperature change between optima. Norman *et al.* (1982) elicited a response within a small temperature range of about 2°C, but they achieved this by rapidly cooling grains *in vitro* on ice for 14 s. Moreover, any further cooling did not increase aleurone response. However, Nicholls (1982) proposed an alternative dual role for temperature in premature α -amylase formation. Ears of the barley variety Himalaya grown at temperatures greater than 20°C in a controlled environment produced immature grains that readily produced α -amylase after desiccation and were classed by Nicholls (1982) as type B. Type A however exhibited a strict requirement for exogenous GA prior to α -amylase secretion, which could suggest it to be less sensitive to GA. The second effect of temperature on premature α -amylase formation becomes apparent during grain drying, post-GA-sensitisation. Lower drying temperatures increase the duration of the lag phase in α -amylase secretion and decrease the initial rate of enzyme production in wheat (Nicholls, 1980). Nevertheless, work by Major (1999), Mrva and Mares (2001a, 2001b and 2002) and results from the current study have demonstrated the potential of a transient high temperature treatment to effect elevated non-sprouting related α -amylase activity in wheat grain *in vivo*. It has been suggested that sensitisation of, and the resulting increase in α -amylase secretion by, the aleurone only takes place after structural re-conformation of cell membrane components (Armstrong *et al.*, 1982; Norman *et al.*, 1982). For any type of temperature treatment to be effective, it would have to bring about these supposed changes in the aleurone membrane.

One possible route through which slow grain drying could affect PMAA formation, is the development of heavier grains. Analysis of experiment CE HA 6 samples revealed that the

number of grains with suspected PMAA increased as grain dry weight increased, both in grain samples exposed to 70% and 85% RH during grain development (Figure 5.9). More importantly however, grains with α -amylase activity in the crease that developed under conditions of high humidity tended to be heavier than grains that matured under the lower humidity regime. Larger grains have been linked to higher α -amylase activity (Evers *et al.*, 1995; Evers, 2000) as there is a greater amount of aleurone tissue contained in the crease region of larger grains, capable of more enzyme secretion (Evers, 2000). Greenwell *et al.* (2001) reported that cells, different from most other aleurone cells, and termed hybrid cells, are only found in crease aleurone. A further investigation into these hybrid cells could give a greater insight into PMAA.

5.5 Conclusion

Experiments carried out on fully controlled environment (CE) grown plants could not substantiate the proposed ability of a three-day transient high temperature period (Major, 1999), during early grain development, to induce PMAA formation in modern UK wheat genotypes. However, circumstantial evidence was produced, suggesting that, for cv. Rialto, there exists a window of susceptibility to a high temperature PMAA stimulus. PMAA induction would however occur during earlier grain development stages than considered during this project.

6.1 Introduction

In this chapter, the goal of developing of a breeders' screen for PMAA is addressed. In order for a screening method to be effective, it firstly requires the ability to allow for an accurate application of an established PMAA environmental trigger at the most susceptible grain development stage. A second requirement is the capacity to screen a large number of varieties or lines. As available CE space limits the number of plants that can be PMAA induced at any time, a method was sought that could facilitate larger numbers. Experiments would be set up to test whether a detached tiller (DT) technique can be used as a means of identifying PMAA susceptibility in UK wheats.

Initially a detached ear (DE) method was considered as a way of applying a specific environmental treatment to grains from a wide range of varieties. The DE technique that was examined is a semi-sterile method for isolating wheat ears on culture medium, described by Donovan and Lee (1977) and later modified by Barlow *et al.* (1983), Singh and Jenner (1983), Gold (1991) and Ahmadi and Baker (2001). The method has been used extensively to study grain physiological processes, as it is assumed that the system produces a good approximation of *in vivo* grain physiology.

Although the DE technique did overcome the problem of limited CE space for applying an environmental treatment to higher numbers, tests proved it to be laborious. A less complex method using DTs was described by Mrva and Mares (2001a) and is an adjustment of a method used by Kato *et al.* (1996). However, unlike detached tillers used in embryo rescue

for the production of double haploid (DH) plants (Inagaki *et al.*, 1997 and 1998), the proposed DT method would have to be capable of sustaining the larger part of grain development, were it to be used in a PMAA screen. DH production requires detached tillers to be artificially supported for only a few weeks starting just prior to anthesis.

As the DT method does away with the need for intricate growth media and sterilisation procedures, the method was further investigated as a potential facet in a PMAA breeders' screen for UK wheat genotypes. α -Amylase detection and PMAA identification would be based on a procedure described by Lunn *et al.* (1996), employing a range of analytical techniques. A working PMAA screening method would make the development of possible molecular markers for PMAA feasible through QTL analysis. Mrva and Mares (2001b and 2002) found two QTLs associated with LMA in Australian wheat. One significant QTL was located on the long arm of chromosome 7B. A weaker QTL with less significant effect was found on the long arm of chromosome 3B.

Due to the nature of the DT method, it was important to demonstrate that the technique can be effectively used with UK phenotypes. Unlike in the DE method, previously used on and proven to work for UK wheats, DT grain filling occurs in an environment depleted of culture supplements. Under stress, leaf compounds are quickly degraded, not only those that serve as reserves, but supposedly also substances found mainly in the chloroplasts (Esquivel *et al.*, 2000; Noiraud *et al.*, 2001). An initial experiment using the DT method was therefore set up to test the hypothesis that DT post anthesis grain filling is dependent on available reserves rather than novel assimilation by the ear and flag leaf in eight winter wheats and one awned spring wheat. Furthermore, demonstrating a similarity in changing grain α -amylase levels in response to environmental cues, between laboratory technique

and conventionally produced grain, would suggest the DT technique to have a high degree of validity as a method for inducing PMAA in susceptible UK genetic backgrounds.

A second parallel experiment to the CE HA experiments, but using the DT method was devised to test the hypothesis that a specific high temperature treatment applied during early DT grain development promotes PMAA formation in susceptible UK winter wheat varieties as well as an LMA susceptible spring wheat.

Work on the DE and DT methods was carried out at Monsanto Cambridge. Plants used for the DT experiments were grown fully in CE chambers, for the same reasons as this was done for experiments with whole plants (chapter 5). As plants grown in the field or glasshouse are exposed to unpredictable and varying conditions, growing plants completely in CE would allow for a much higher degree of control over the plants' environment. Variation in the grain characteristics of interest could then more readily be associated with certain environmental parameters. This is especially important when it comes to potential differences in PMAA expression. Differences between a control group and plants exposed to a specific environmental treatment could more confidently be attributed to the treatment, as the background environment would have been similar for all plants.

6.2 Materials and methods

6.2.1 Varieties

In addition to the three winter wheat varieties Haven, Option and Rialto, used for the CE experiments with whole plants, six genotypes were selected for tests and an experiment with the DT method. These genotypes were Fenman, Malacca, Maris Huntsman, Monsanto

line 00/7, Potent and Seri. The varieties were chosen because of their suspected susceptibility or resistance to PMAA and their differences in HFN and sprouting score. Maris Huntsman is a variety prone to PMAA (Gale *et al.*, 1983; Greenwell *et al.*, 2001) and was used in early work identifying the problem. The susceptibility was most likely inherited (Bingham and Whitmore, 1966) from the parental line Professeur Marchal (Anonymous, 1991). Fenman is also thought to have a predisposition to PMAA (Gold and Duffus, 1996) and is in the Professeur Marchal lineage through cv. Hobbit. On the other hand, Malacca was added because of its group 1 characteristics (HFN 312 s and sprouting resistance 6) (Anonymous, 2003) and suspected resistance to PMAA. Although not on the recommended list of winter wheats, the variety Potent was chosen because of its low HFN in the presence of a high sprouting score, similar or even higher than the score for Rialto. In addition, a promising line designated MC 00/7 from the Monsanto (UK) Ltd. breeding program was also added, as was the spring wheat Seri, prone to LMA in Australia. Hagbergs and sprouting and PMAA scores are given in Table 6.1.

Table 6.1 HFN, sprouting scores and PMAA susceptibility for eight winter wheats^w and one spring wheat^s

Variety/line	HFN	Sprouting score	PMAA
Fenman ^w	~	~	+
Haven ^w	low-medium	2	+
Malacca ^w	312 s	6	- suspected
Maris Huntsman ^w	~	~	+
MC 00/7 ^w	~	~	+ suspected
Option ^w	277 s	8	-
Potent ^w	165 s	6/7 unofficial	+ suspected
Rialto ^w	220 s	6	+
Seri ^s	~	~	+

Hagberg falling number (HFN) is expressed in seconds or in three classes, low-medium-high.

Higher sprouting scores indicate an increased resistance to sprouting. + PMAA susceptible, - not prone to PMAA. Figures are from NIAB recommended lists or Monsanto Cambridge

6.2.2 Preliminary method development

6.2.2.1 Detached ears

Ear isolation was a modification of a technique used by Ahmadi and Baker (2001). Stems bearing ears were cut below the penultimate node from the ear and surface sterilised using a sodium hypochlorite (Sigma-Aldrich) solution with 0.5% available chlorine. Ears were wrapped in cling film and stems reduced to 10-15 cm. The cut was made diagonally with a scalpel under sterile distilled water in a clean area of the lab. Ears were immediately placed in a tray with sterile distilled water in a horizontal laminar airflow cabinet, with the ears pointing away from the filter wall. Stems were again shortened by approximately 2 cm using the previous procedure and placed in test tubes containing culture medium by fitting them through a sterile cotton plug sealing the vessels. The DEs and cotton plugs were secured with pierced cling film or aluminium foil. Cling film was removed from the ears and the test tubes were wrapped in aluminium foil (reflective surface facing outwards). Test tubes were placed in a water bath containing deionised water (section below) circulated at 5°C (Ahmadi and Baker, 2001), in order to suppress potential microbial growth in the culture medium. The water bath was situated in a CE chamber (Weiss Technik, Germany) (see section 6.2.3).

Culture medium preparation and composition of major stock solutions was according to Donovan and Lee (1977). Minor elements, iron and vitamin supplements were made as described by Linsmaier and Skoog (1965), and were an adaptation of the standard MS macro, micro and vitamin supplements (Murashige and Skoog, 1962), but contained only the essential vitamins myo-inositol and thiamine·HCl. An amino acid solution as a source of nitrogen was prepared as described in Donovan and Lee (1977), but only included

glutamine (0.02 M) (Ahmadi and Baker, 2001). As all leaves including the flag leaf were removed and stems were greatly reduced in length it was decided to also add a relatively large amount of sugar to the solution, as this would serve as an additional, medium derived, source of carbon during grain development. The sucrose concentration was increased to 116.9×10^{-3} M (40 g/l) (Barlow *et al.*, 1983), double the concentration used by Donovan and Lee (1977). The culture medium was filter sterilised using a ≤ 0.45 μm filter (Millipore (UK) Ltd., Watford, Hertfordshire).

Tillers with mid-ear grains (section 5.2.2) at approximately ZGS 75 were cut, stubs wrapped in damp tissue paper and immediately moved to the laboratory. A total of 36 ears were isolated and placed in the water bath. The CE chamber housing the water bath with DEs was maintained at 20°C during a 20 h light period and at 15°C during the dark period. The RH was set to 75%.

Soon after isolation however signs of infection developed in the ears. A later investigation revealed the disease to already have been present in plants from which the DEs were isolated. An emerging severe mildew infection ultimately made harvested grain unsuitable for analysis. A later attempt to have a clean crop grown under controlled field or glasshouse conditions was abandoned due to practical reasons. These included a lack of available resources and space to implement appropriate phytosanitary measures.

6.2.2.2 Detached tillers

The development of the DE method as part of a breeders' screen for PMAA was no longer pursued as the DT method was deemed to be more suitable. It offers the prospect of simpler and faster treatment of a larger number of entries.

Ears were tagged at full ear emergence (ZGS 59), and at various subsequent development stages, stems were cut with sharpened scissors as close to the ground as possible without picking up any soil. Stems were cut diagonally with a flamed scalpel dipped in 70% ethanol, 2 to 3 cm below the third node from the ear. Tillers retaining only the flag leaf were placed in a water bath filled with deionised water in a CE chamber (Mrva and Mares, 2001a and 2001b) maintained at 60% RH. Deionised water was circulated through the water bath and maintained at 10°C. Of each plant only the five primary tillers were used as DTs. Grain was harvested after full ear senescence (ZGS >92).

As grains harvested from DT tests and experiment CE M 1 (section 6.2.6) were found to be shrivelled, 16 DTs of cv. Rialto grown in controlled environment cabinets at Harper Adams were cut and placed in conical flasks (Plate 6.1), in order to investigate the cause of the underdevelopment. Considerable dye absorption would suggest that shrivelling is not due to a lack of water uptake. The flasks contained a solution consisting of 196 ml deionised water and 4 ml of carmoisine (E122) stock solution (Cochineal Food Colouring (artificial), Supercolor, Leeds). Flasks with DTs were returned to the CE cabinet for 48 h. The cabinet was maintained at 20°C during a 16 h day and 15°C during the dark period. The relative humidity was lowered from 75% to 60%.

Plate 6.1 Rialto DTs on red dye solution consisting of deionised water and carmoisine



6.2.2.3 Water bath

As most water baths on the market have poor cooling and temperature regulatory capabilities, and will hold only a small number of test tube racks (DE method), or a limited number of DTs, purpose-built water baths were constructed at Monsanto Cambridge. In the Weiss CE chambers (see below), a stainless steel tank (Grant Instruments (Cambridge) Ltd, Shepreth, Cambridge) was supported on a metal framework (Plate 6.2 *left*). Water temperature in the tank was controlled by a flowheater (Grant Instruments (Cambridge) Ltd, Shepreth, Cambridge) with accurate lower and upper temperature limit regulation. The flowheater was placed behind an accompanying cooling unit relative to the direction of water circulation. A grid was positioned a few centimetres above the waterline. A second grid was adjustable to 80 cm above the waterline (Plate 6.2 *Right*).

Plate 6.2 *Left* Equipment used in the construction of a DE/DT water bath, *Right* Initial tests with detached tillers



Left Stainless steel tank (top), flow heater (bottom left) and cooling unit (bottom right) (Grant Instruments Ltd.)

Water baths used in a clean area (next section) were of a similar construction, but the tanks were custom built on-site from moulded plastic and were highly insulated with >2 cm of foam. These tanks were easier to handle and keep clean.

All tanks were cleaned by circulating commercially available non-toxic disinfecting detergents through the tanks for 12 h and rinsing three times with deionised water.

6.2.3 Growing plants in a controlled environment

Plants used for testing the DT method were grown in CE chambers (Weiss Technik, Germany) at Monsanto Cambridge. Additional test plants were grown in a nearby refrigerated glasshouse maintained at similar conditions as the CE chambers (see below). The glasshouse plants and plants grown in the Weiss CE chamber suffered from the same diseases as plants used in CE experiments at Harper Adams. Both vernalisation and vegetative growth of plants used for a final DT experiment was therefore done in CE chambers (Convion, Canada) situated in a clean laboratory area, maintained at a higher air pressure than its surroundings by continuous introduction of filtered air.

Grains were given a micro dressing containing carboxin and thiram as described in section 5.2.2. Treated grains were sown in plastic trays in Levington F1 (Scotts UK, Ipswich) compost and germinated at 10°C in a CE room. At ZGS 12 seedlings were moved to another CE chamber maintained at 4°C during an 8 h light period and 80-90% RH for 10 weeks. Vernalised plants were potted in 2 l pots in Levington C2 (Scotts UK, Ipswich) compost and moved to a CE chamber maintained at 20°C for a 16 h photo period and 15°C during the dark period. Relative humidity in the clean Conviron CE chambers was maintained at 60%, while in the Weiss CE chambers RH was kept at 70% to reduce the number of times a chamber needed to be entered for watering. Between crops the Weiss CE chambers were given a crude decontamination by washing the inside walls with warm water and leaving the chamber to run empty for five days at 30°C and > 90% RH.

Lighting in the Weiss CE chambers was provided solely by high-pressure sodium lamps, while lighting in the Conviron came from metal halide lamps and Edison screw light bulbs. Light intensity was measured as described in section 5.2.2.2. The PAR flux in the Weiss CE chambers was 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at approximate ear height, while the light intensity in the Conviron was set to 750 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR.

Due to health and safety considerations, maintenance of the CE chambers was strictly the responsibility of Monsanto (UK) Ltd. staff.

6.2.4 Agronomy

All plants were watered with tap water at the base to minimise the risk of disease. Between the end of stem elongation and ear emergence plants were given approximately 3 g of NPK

(12.4% 11.4% 17.7%) fertiliser granules (Hydro, Norway). Where applicable, a fungicide application was given as described in section 5.2.2.1.

6.2.5 Grain analysis

α -Amylase activity (chapter 3) was determined for 30 grains from each ear. Visual assessment of sprouting and total α -amylase activity measurements were carried out as described in section 3.2. Due to the large number of samples however a Cemotec Sample Mill 1090 (FOSS Tecator, Sweden) was used to mill the samples. Between samples the mill was thoroughly cleaned with a fine brush and vacuum cleaner.

Five samples taken from a high HFN Rialto crop were each split into two groups. One group was sprouted for 72 h (section 4.2.1). The ten sub-samples were again divided into two groups. One sub-group of grains was milled using the Cemotec mill, while the other was processed with a pestle and mortar. No significant difference in α -amylase activity was found between the two procedures. In addition, no significant difference in α -amylase activity could be found between non-sprouted samples milled on a clean mill and non-sprouted samples milled directly after sprouted samples, separated only by the between samples cleaning process. This suggests that no sample-to-sample contamination took place.

In addition to pre- and post-harvest grain measurements, post-harvest stem dry weight (method described in section 5.2.3) was determined on earless stems cut directly below the third node from the ear. Post-harvest stem dry weight and flag leaf length were used in the analysis of grain data (section 6.2.7, results in 6.3.2).

6.2.6 Experimental design

The experiments with DTs were of a fully randomised design. Plants were repositioned periodically and tillers were cut within a narrow range of milk development. Areas in the growth chamber that were clearly different with regard to light measurement and temperature, from the rest of the chamber, were not used for growing plants.

The first DT experiment CE M 1 was set up to test the effect of stem length and presence or absence of the flag leaf on grain filling in detached tillers. In addition to grain weight, the number of physiologically mature grains (Hanft *et al.*, 1982) and the ability of grains to germinate would be assessed. However, plants grown for CE M 1 were lost, together with plants from their sister experiment CE HA 2 due to a fault with the CE equipment and loss of power to the water bath set up.

Experiment CE M 2 carried out in the clean CE chambers, examined the effect of a three day high temperature treatment devised by Major (1999) on PMAA formation in DTs isolated during the milk development stage. DTs were cut and transferred to water baths in either a 20/15°C or 26/20°C chamber, when grains in the mid-ear were at ZGS 74-77 (section 5.2.2). After treatment, all DTs were placed in the water bath in the 20/15°C CE chamber. Grains were harvested when tillers had fully senesced (ZGS >92). A skeleton ANOVA is given in Table 6.2.

Table 6.2 Skeleton ANOVA for CE M 2

Source of variation	df
Variety	8
Treatment	1
Variety × Treatment	8
Error	162
Total	180-1=179

6.2.7 Statistical analysis

Analysis of variance, without and with adjustment for covariates, and regression analysis, including linear regression in groups, were carried out in Genstat for Windows 6th edition. Data was checked for the nature of the distribution of residuals, but most likely due to the larger number of data points compared to CE HA experiments transformation proved not to be necessary for DT data.

6.3 Results

6.3.1 Assessment of DT water uptake

Of 16 Rialto DTs isolated on carmoisine solution all displayed a visible red tinge within a short period of time. Within several hours the presence of red dye became noticeable in the tops of the ears. The colour deepened, until after 48 h all outer lemmas and flag leaves showed conspicuous dye uptake (Plate 6.3 *Left*). Distribution of the dye was symmetrical in transverse stem sections (Plate 6.3 *Right*) over the full length of the stem, indicating the absence of embolisms or other obstructions in the xylem, or the presence of an effective compensatory mechanism.

Nevertheless, most grains harvested from CE M 2 were shrivelled. None of the grains showed any visible signs of sprouting.

Plate 6.3 *Left* Rialto DT after 48 h on a carmoisine solution, *Right* red dye evenly colouring the xylem of the flag leaf node



Bar corresponds to 1 mm

6.3.2 Grain dry weight in relation to other grain characteristics

Light intensity for CE M 2 was higher than for DT tests and CE M 1 and it was therefore anticipated that grains would be better developed. Salisbury *et al.* (1987) had shown that an increase in CE PAR from 400 to 1700 $\mu\text{mol m}^{-2} \text{s}^{-1}$ is accompanied by an almost linear increase in wheat biomass production. However, grain of CE M 2 was also shrivelled. A possible lack of available nutrients as cause for the shrivelling was examined by looking at the relationship between post-harvest stem dry weight (Table 6.4) as an indication of available reserves and grain weight (Figure 6.1). While a weak but significant positive linear trend ($R^2 = 0.115$, $P < 0.001$) was found between DT stem dry mass and the mean grain dry mass when analysing the combined data, an apparently near horizontal slope, though not significant, was found for the varieties separately.

The number of grains in an ear has been linked to grain weight and it has been shown that the environment can have had an effect on both final number and weight (Abbate *et al.*, 1995 and 1997). Ears with fewer grains tend to produce heavier grains. The mean grain dry

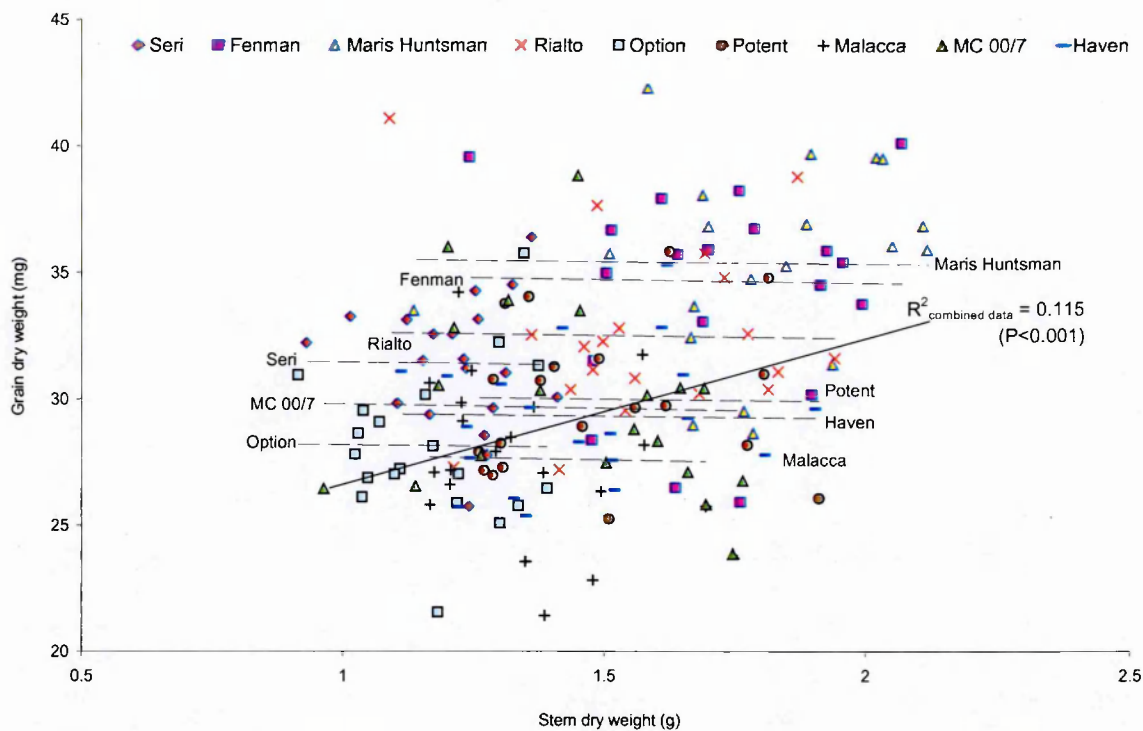


Figure 6.1 Relationship between mean grain dry mass per tiller and dry mass of the DT stem

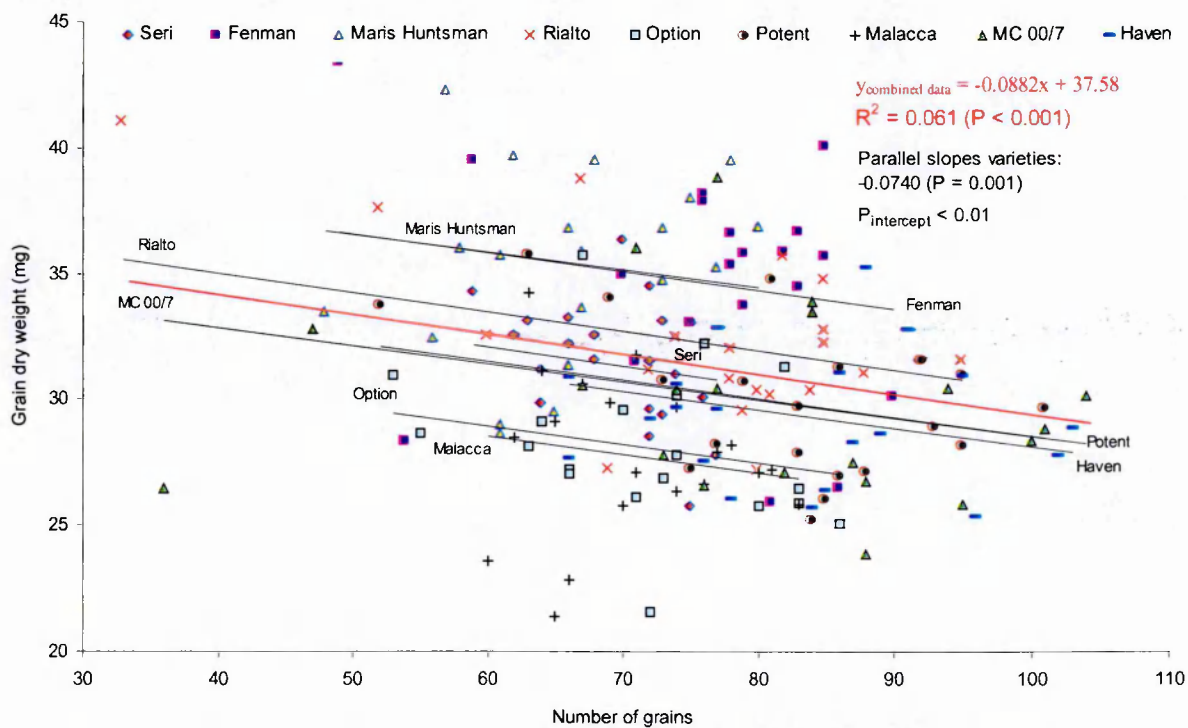


Figure 6.2 Relationship between mean grain dry mass and the number of grains per ear

mass was therefore also examined in relation to the number of grains in the ears (Figure 6.2). Similar near horizontal slopes were found for both individual varieties, -0.074 ($P = 0.001$) and the combined data, -0.0882 ($P < 0.001$). In spite of a negative trend, with grain weight increasing as the number of grains in an ear decreases, the near horizontalness of the relationship suggests that grain number had no effect on grain dry mass deposition in the detached tillers.

It was thought that photosynthesis could have continued to take place in the DTs, given that both ears and flag leaves remained green for a considerable period of time. Flag leaf length (Table 6.4) as an indicator of assimilation potential however bore no significant linear relationship with grain dry mass (Table 6.6).

6.3.3 α -Amylase analysis

DT α -amylase measurements (chapter 3) for CE M 2 were relatively high. Although the activity of Rialto and Option was just above activities of field (Rialto, chapter 4) or CE (Option and Rialto, chapter 5) grown grain samples with no or low sprouting, Haven had many times the activity (Table 6.3) measured in CE experiments with whole plants.

Fenman and Maris Huntsman however far exceeded the other varieties, with α -amylase activities several times higher than those found for Haven.

As expected, ANOVA revealed a significant ($P < 0.001$) difference in α -amylase activity between the varieties. On the other hand, the high temperature treatment had no significant overall effect on activity, but a significant ($P < 0.001$) treatment \times variety interaction was found. Interestingly, while Seri exhibited a large difference in α -amylase activity, with the high temperature treatment activity 1120 mEU/g dw higher than the 2107 mEU/g dw of the

Table 6.3 Mean α -amylase values for a high temperature DT experiment with nine wheat lines

	mEU/g dw			mEU/g dw			mEU/g dw	
Fenman	Control	3621	Maris Huntsman	Control	4655	Potent	Control	226
	Treatment	3861		Treatment	4296		Treatment	230
	Mean	3741		Mean	4476		Mean	228
Haven	Control	2120	MC 00/7	Control	406	Rialto	Control	260
	Treatment	992		Treatment	580		Treatment	219
	Mean	1556		Mean	493		Mean	240
Malacca	Control	66	Option	Control	95	Seri	Control	2107
	Treatment	71		Treatment	118		Treatment	3227
	Mean	68		Mean	107		Mean	2667
Variety L.S.D.	431.3		Treatment L.S.D.	203.3		Treatment×Variety L.S.D.	610.0	
P value	<.001		P value	0.966		P value	<.001	

Control: maintained at 20°C/ 15°C, Treatment: transfer to 26°C/ 20°C for 3 days during milk development.

Sample size 30 grains.

control mean, Haven produced an opposite pattern. The Haven control mean was 1128 mEU/g dw higher than the high temperature treatment mean of 992 mEU/g dw.

Results from chapter 5 suggested that, for cv. Rialto at least, an earlier grain development stage than previously thought might be more appropriate for the high temperature induction of PMAA. No significant linear relationship was however found between DT α -amylase activity (Table 6.3) and the percentage grain moisture at treatment transfer (Table 6.4). This finding could be due to the fact that DTs were cut within a narrow grain development range, with an overall mean and s.d. of 71.6 ± 2.08 % grain moisture.

Table 6.4 Mean percentage grain moisture at DT isolation and post-harvest flag leaf length and stem dry mass, for nine lines used in the DT experiment

	% grain moisture	s.d.	S.E.M.	flag leaf length (cm)	s.d.	S.E.M.	Stem dry weight (g)	s.d.	S.E.M.
Malacca	72.0	2.41	0.54	18.4	2.56	0.57	1.34	0.155	0.035
Option	70.6	3.54	0.79	28.5	5.74	1.28	1.17	0.138	0.031
Haven	72.1	1.72	0.38	15.5	3.55	0.79	1.45	0.213	0.048
MC 00/7	72.4	1.21	0.27	18.9	3.43	0.77	1.45	0.232	0.052
Potent	70.2	1.43	0.32	17.4	4.07	0.91	1.49	0.209	0.047
Seri	71.0	1.06	0.24	15.1	3.20	0.71	1.22	0.114	0.026
Rialto	71.1	2.21	0.49	19.6	4.38	0.98	1.58	0.222	0.050
Fenman	72.5	1.20	0.27	27.2	5.05	1.13	1.70	0.221	0.050
Maris Huntsman	73.0	0.97	0.22	22.7	6.57	1.47	1.80	0.235	0.052

Stem dry weight was determined on earless stems cut directly below the third node from the ear. Varieties are arranged top-down according to increasing post-harvest grain dry matter means (Table 6.6).

Work by Evers *et al.* (1995) and Evers (2000) suggests that PMAA susceptibility is linked to grain size (see section 2.4.2), most likely through the effect increased grain growth has on crease aleurone morphology and physiology. Hence α -amylase activity was analysed in relation to post-harvest grain dry mass as a measure of grain size. A weak but significant linear relationship was found between mean grain dry mass and the α -amylase activity of ears (Figure 6.3). Regression for individual varieties on the other hand did not expose any significant linear association between the two parameters.

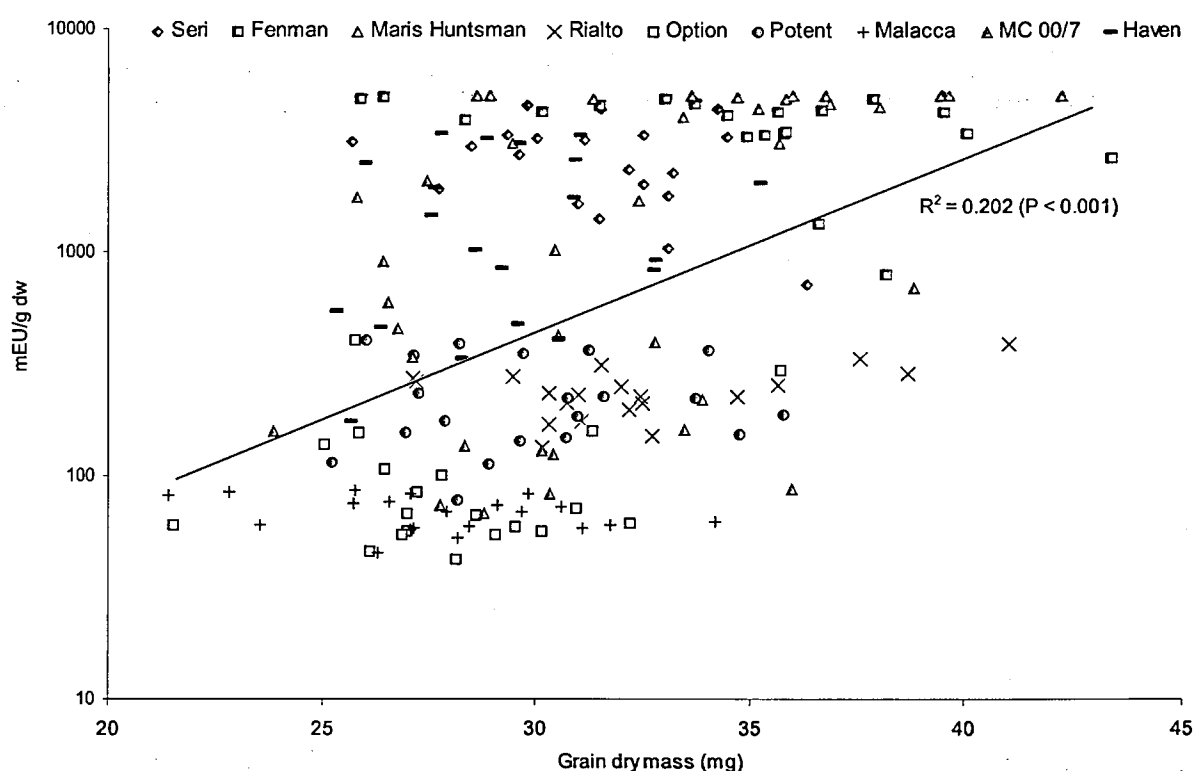


Figure 6.3 Relationship between α -amylase activity of harvested grain and mean grain dry mass

Given also the *a priori* implication of a link between grain size and enzyme activity, α -amylase activity was adjusted for grain dry mass as a covariate. The procedure increased the difference between the control mean and the treatment mean for the varieties in general, when the smaller of the mean α -amylase measurements was subtracted from the larger. The range in mean varietal α -amylase activity was also increased from 4408 mEU/g dw to 4645 mEU/g dw. ANOVA of α -amylase activity adjusted for the covariate, revealed

not only a significant varietal and treatment \times variety interaction effect, but in addition to the non-adjusted data (Table 6.3) also a significant effect of treatment (Table 6.5). However, this significant effect of treatment was not an increase in α -amylase activity relative to a control group, as reported by Major (1999), but rather the opposite. Although Seri did again displayed a large difference in α -amylase activity, with the treatment mean 1092 mEU/g dw higher than the control, Haven showed a large but opposite difference in activity with the control mean 1193 mEU/g dw higher than the treatment mean. Moreover, although Fenman, MC 00/7 and Seri displayed a higher mean activity for the high temperature treatment than the control, for the majority of the varieties this pattern was reversed, with high temperature treatment reducing the amount of α -amylase present in the harvested grain.

Table 6.5 Mean α -amylase values adjusted for average grain dry mass as covariate, for a high temperature DT experiment with nine wheat lines

	mEU/g dw			mEU/g dw			mEU/g dw	
Fenman	Control	3801	Maris Huntsman	Control	4875	Potent	Control	199
	Treatment	3916		Treatment	4347		Treatment	193
	Mean	3859		Mean	4611		Mean	196
Haven	Control	2099	MC 00/7	Control	377	Rialto	Control	326
	Treatment	906		Treatment	534		Treatment	249
	Mean	1503		Mean	456		Mean	288
Malacca	Control	0	Option	Control	50	Seri	Control	2135
	Treatment	-68		Treatment	-15		Treatment	3227
	Mean	-34		Mean	17		Mean	2681
Control mean		1540	Treatment mean		1477	P value Covariate		0.068
Variety L.S.D.		447.4	Treatment L.S.D.		214.6	Treatment \times Variety L.S.D.		622.0
P value		<.001	P value		<.001	P value		<.001

Control: maintained at 20°C/ 15°C, Treatment: transfer to 26°C/ 20°C for 3 days during milk development.

Sample size 30 grains.

Not entirely unexpectedly, the varieties with the lowest grain dry weight also produced the lowest average α -amylase activity and the heaviest grained varieties also produced the largest amount of grain enzyme (weights in Table 6.6). While Malacca and Option had an unadjusted mean α -amylase activity of 68 and 107 mEU/g dw respectively, the heavier

Maris Huntsman and Fenman produced comparatively copious amounts of α -amylase enzyme, averaging 4476 and 3741 mEU/g dw correspondingly.

Table 6.6 Mean post-harvest grain dry mass for nine lines used in the DT experiment

	dry mass (mg)	s.d.	S.E.M.
Malacca	27.7	3.10	0.69
Option	28.1	3.04	0.68
Haven	29.3	2.59	0.58
MC 00/7	29.8	3.72	0.83
Potent	30.0	2.98	0.67
Seri	31.4	2.52	0.56
Rialto	32.5	3.57	0.80
Fenman	34.7	4.49	1.00
Maris Huntsman	35.3	3.75	0.84

6.4 Discussion

Even though grains from the detached tiller experiment were shrivelled, varieties with heavier grains tended to produce higher α -amylase activities (Figure 6.3). This could appear to support the suggestion by Evers *et al.* (1995) that α -amylase activity in grains is more likely to increase as grain size increases. According to them, larger grains tend to have a larger crease surface area and could therefore hold a greater amount of (crease) aleurone tissue. In addition, Greenwell *et al.* (2001) reported the presence of cells in crease aleurone with an abnormal morphology and degree of differentiation, as well as cells with a tendency to produce α -amylase activity under unusual circumstances. In contrast to results from the DT experiment, which suggest a varietal difference in grain dry matter content rather than a relationship with the number of grains per ear (Figure 6.2), other studies have shown that individual grain weight tends to increase with a reduction in the number of grains per ear. Abbate *et al.* (1997) found that kernels per unit spike dry matter declined with increasing spike weight. Abbate *et al.* (1995) suggested that a nitrogen-linked mechanism might be operating through the survival of differentiated flowers.

According to Bindraban *et al.* (1998), during a development phase ending a week after anthesis, floret maturation and death takes place. This is followed by ovule fertilisation or grain set, resulting in the final number that will develop to become immature grains. The *Rht*-gene has a wide sphere of influence and affects various plant and grain morphological and physiological traits. *Rht* has also been implicated in the reduction of PMAA expression (section 2.4.4). It is therefore interesting to note that Miralles *et al.* (1998) reported the number of grains per spike to be significantly greater in NILs with *Rht* alleles than in standard height lines, suggesting that *Rht* might influence PMAA physiology through a mechanism other than grain number- and size-regulation. Increases in maximum temperature during a five-day period ending at 50% anthesis lowered the number of grains per ear at harvest maturity, for the winter wheat variety Hereward (Wheeler *et al.*, 2000). This raises the question, if high temperature PMAA induction and final PMAA levels might be the result of additive effects of various (physiological) factors. Transient high temperature treatment at pre-milk grain development stages (ZGS <71) should be considered in future studies.

The absence of visible signs of sprouting is a strong indication that the high α -amylase measurements found for the DTs are not due to germination enzymes. Although PMAA could form part of the exceptionally high DT α -amylase activity, it can not be ruled out that a major share of the activity is as a consequence of developmental α -amylase retention, or abnormal α -amylase secretion initiated while grains were still immature. A change in DT water influx after isolation could have triggered a physiological response. Cornford *et al.* (1987b) found that normal responsiveness to gibberellin is attained as a result of grain maturation, but that it could also be induced in immature non-GA-responsive grains by drying treatments (Armstrong *et al.*, 1982). Although GA levels are relatively low in mature dry grains, during grain development, endosperm and aleurone

contain many times the amount of GA present in mature grains (Jacobsen and Chandler, 1988). Sensitisation to GA during development, perhaps prior to a potentially protective ABA peak, could have initiated excessive enzyme secretion. Other sources of α -amylase not normally found in mature grains have been described by Gale and Ainsworth (1984) and Baulcombe *et al.* (1987) and include AMY2 and AMY3 isozymes. An adjustment of the DT method might be necessary, depending on further examination of water uptake by DTs. DT water economy could have been affected by the loss of root pressure, the isolation technique or even microbial growth. Although tests with Rialto DTs have shown that uptake of water occurs apparently unhindered up to 48 h after isolation, it can not be ruled out that longer-term microbial growth can potentially obstruct flow through the vascular system. It can also not be ruled out that grains stopped depositing dry matter altogether, after DTs were cut. No temporal assessment of grain dry matter deposition was however made during this study, as it was felt that this would alter the development of the remaining grains (see section 2.4.2) in the detached ears. The fact that, in general, α -amylase activities in controls were similar to or higher than activities found for high temperature treated DTs (Table 6.3) (even more so when grain dry mass was used as a covariate in the ANOVA (Table 6.5)), suggests that higher temperatures could have hastened grain drying in the high temperature treated DTs, therefore preventing α -amylase to accumulate to the same post-harvest levels as found for the control tillers. Thus, a combination of temperature regime and drying conditions would have acted in concert to produce these findings. It is not clear from the literature whether there is a dominant PMAA stimulus, or if high temperature treatment and grain drying augment each other. Or even, if they can function antagonistically. Plants grown for DTs and the DTs themselves, were kept at an RH that was 10-25% lower than the humidity used in experiments with whole plants (chapter 5).

Both grains grown under $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $750 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR were shrivelled. It is therefore thought that limited photosynthesis due to reduced radiation is not a primary cause of poor dry matter deposition, assuming that water uptake was not inhibited. Poor grain filling could nevertheless have been due to a lack of available metabolites and larger DTs could have produced a better grain yield. The presence of primary photosynthesis carbohydrates in tissues of a range of plant species lacking the synthesis enzymes clearly demonstrates long-distance carbohydrate transport from the major photosynthesis organs, the leaves, to other organs (Noiraud *et al.*, 2001). Similarly to carbohydrates, Esquivel *et al.* (2000) reported that nutrient stress can result in the degradation of storage or water soluble proteins in the leaves, thus re-releasing organic nitrogen as a mixture of amino acids. More importantly however for DTs, stored stem reserves serve as a source of carbon for grain filling in wheat, particularly during conditions of stress. Cruz-Aguado *et al.* (2000) found that in wheat grown in warm climates, the second internode from the top supplied the larger part of non-structural carbohydrates to the grain compared to the other internodes. Nitrogen requirement for protein synthesis in the developing grain is offset by mobilisation of earlier assimilated N present in vegetative tissues and by direct uptake and assimilation of N during grain filling (Mi *et al.*, 2000), but with re-mobilised N suggested to be the major source of nitrogen to the grain (Austin *et al.*, 1977). In their studies Austin *et al.* (1977) found that during grain filling, plants which lost the most dry weight from their stems and leaves took up the least nitrogen *in vivo*. Singh and Jenner (1983) found that some ears grown on a culture medium with an added amino acid mixture would produce grains that were heavier than grains from a field grown crop of the same variety. Results from the CE M 2 experiment with DTs suggests that larger tillers hold more reserves which can be mobilised during filling, since a positive linear relationship was found between stem dry weight and grain weight. As the DT method was partly chosen for its potential ability to grow grains in the absence of complex growth media, further work

on the method should focus on growing heavier DTs, possibly under strict phytosanitary measures in the field. It remains to be seen if nutrient stress had an impact on α -amylase formation.

6.5 Conclusion

Although DT grains failed to develop normally during the current study, successive adjustment of the method, making it suitable for culturing DTs with the intention of harvesting viable grain, should make more detailed investigations of PMAA physiology possible.

The occurrence of a predisposition to pre-maturity α -amylase activity (PMAA) in modern UK winter wheat varieties has clearly been demonstrated during this project, with the identification of PMAA in field grown cv. Rialto. In addition, using information gathered from the Rialto crops and from controlled environment (CE) experiments with whole plants and detached tillers (DTs), a significant step has been made towards identifying a robust stimulus for PMAA in a range of wheat genotypes. An effective stimulus would consist of a combination of environmental factors with the potential to consistently induce and influence the accumulation of grain α -amylase in the absence of sprouting, in those genotypes that are predisposed to PMAA.

Work by Major (1999) suggests that a high temperature treatment during milk development is more inclined to induce pre-mature α -amylase formation in the grain than a similar high temperature treatment during the later dough development stage. Results from the current study indicate that the most susceptible stage for PMAA induction could be even earlier than suggested by Major (1999). Previous work has suggested that grain drying can also sensitise aleurone to GA and hence elicit pre-mature α -amylase secretion. While studies (chapter 5; Major, 1999) have shown that a high temperature treatment *in vivo* can trigger pre-mature α -amylase production in grains of which the majority of the mass is still water, laboratory drying experiments by Armstrong *et al.* (1982) showed that de-embryonated grains were totally incompetent to respond to external GA unless water content was reduced below 25-30%. This difference in grain moisture content between high temperature and drying induced pre-mature enzyme secretion suggests the high temperature and drying stimuli to be separate phenomena affecting PMAA physiology.

Results from CE experiments with whole plants show that by and large grain moisture *in vivo* is clearly linked to grain development stage (section 5.3.3). However, both high temperature and drying have been suggested to induce enzyme production through changes in aleurone cell membranes. In addition to the proposed temperature dependent structural re-conformation of cell membrane components (Armstrong *et al.*, 1982; Norman *et al.*, 1982), it has been suggested that drying and re-imbibition may cause a structural change in a cell membrane system, possibly a transitory alteration of bilayer spacing (Seewaldt *et al.*, 1981). On the other hand, even if temperature would not act as a stimulus on its own, it can still have a modifying effect on enzyme production. In grain drying experiments Nicholls (1980 and 1982) found that low temperature increased the duration of the lag phase in the subsequent aleurone response to gibberellin. Furthermore, environmental pressures that hasten the reduction of grain moisture content past 10% (Armstrong *et al.*, 1982), could curb α -amylase accumulation in the grain. Gold and Duffus (1996) and Major (1999) had proposed that a transient high temperature period be followed by an increase in relative humidity, as a subsequent extension of the grain drying period would allow for more α -amylase to accumulate. Additional work is required with a combination of high temperature treatments during different grain development stages and various levels of relative air humidity during grain filling, in order to determine an optimum environmental stimulus for PMAA in modern UK winter wheat genotypes.

Some results from the current study, and work by Major (1999), suggest that a high temperature treatment during grain development can induce pre-mature α -amylase formation. In contrast, Mrva and Mares (2001a) have demonstrated the ability of a cold treatment to induce non-sprouting related α -amylase formation. Norman *et al.* (1982) suggested the existence of a temperature shock treatment with upper and lower threshold temperatures *in vitro*, with regard to acquired aleurone sensitivity to GA. Nicholls (1982)

on the other hand, proposed that different temperature regimes during grain filling modify α -amylase production and accumulation. Further work with transient high temperature as a stimulus for PMAA formation should take into account, the temperature history of the grain, the magnitude of the high temperature treatment, and subsequent temperatures during the remainder of grain filling *in vivo*. Temperature history and genotype could affect the time of onset, or alter the length, of a window of susceptibility to a high temperature treatment. This could partly explain why some data derived from both field (chapter 4) and CE (chapter 5 and 6) experiments implied that a high temperature period during early grain development could lower PMAA (also see section 6.4, 2nd paragraph). A robust PMAA stimulus that would induce pre-maturity α -amylase formation reliably and consistently will most likely be genotype specific, and could be a specific transient high temperature period linked to a particular temperature history of plant and/or grain.

Enzyme activity in DTs grown during the current study (chapter 6) was exceptionally high compared to activity found in field-grown material (chapter 4). High activities in samples of the latter could be as a result of α -amylase activity in just a few grains (section 4.3.2). Conversely, any environmental factor promoting developmental α -amylase formation in the CE grown tillers would have affected a far greater number of grains as plants were highly uniform and DTs were cut within a narrow developmental range. It is therefore thought that the majority of grains would have been equally receptive to aleurone sensitisation, though further tests on individual grains are required for definite confirmation.

An adjustment of the DT method might not prove essential with regard to the requirement for additional nutrient supplements (discussed in previous chapter), but a separate further examination should be made of water uptake by DTs. Although tests have shown that

uptake of water occurs apparently unhindered up to 48 h after isolation (section 6.3.1), there is the possibility that longer-term microbial growth does take place, to such an extent that it could cause potential obstructions in the xylem. Unlike Mrva and Mares (2001a), Kato *et al.* (1996) added sulfurous acid to the water bath to suppress microbial growth and they cut back the stems periodically to avoid decay of the ends.

In addition to the development of a working DT method, as part of an effective breeders' screen for PMAA, fast and accurate identification of pre-mature α -amylase is highly desirable. When PMAA is induced in CE in the absence of sprouting, clear identification should not be difficult. However, the ability to measure PMAA in other material, potentially containing some sprouting in the grain, would be of great advantage to industry. Further tests identifying the location and nature of grain α -amylase could help separate PMAA from germination α -amylase in grain fractions or sub-samples. Although Gale and Lenton (1987) reported that pre-maturity α -amylase production is initiated and accumulated more rapidly at the embryo end of the grain, Cornford *et al.* (1987b) observed clear ribbons of activity throughout the crease region of longitudinally halved grains, in the absence of significant scutellar activity. Adjusting the presently used combination of analytical techniques (Lunn *et al.*, 1996) for PMAA identification should also be considered. A prospective fast and sensitive α -amylase FRET assay (Greenwell *et al.*, 2001) could potentially replace the current microtitre assay. A substantial improvement in PMAA detection could be made with the development of a quantitative version of an α -amylase ELISA (Skerritt *et al.*, 2001) used by Mrva and Mares (2001b and 2002) to identify high pI isozymes.

This project has added to what is known about PMAA thus far. However, a great deal more work needs to be done before a robust PMAA-stimulus can be identified with confidence.

The development of a detached tiller technique, towards which a start has been made during this project, should not only aid research into PMAA, but should help develop a PMAA screening method. The development of an effective screening method for PMAA would be highly advantageous to both UK wheat breeders and farmers. It would add another tool to the arsenal of methods that has become available in recent times, to control the breadmaking potential of home-grown wheats.

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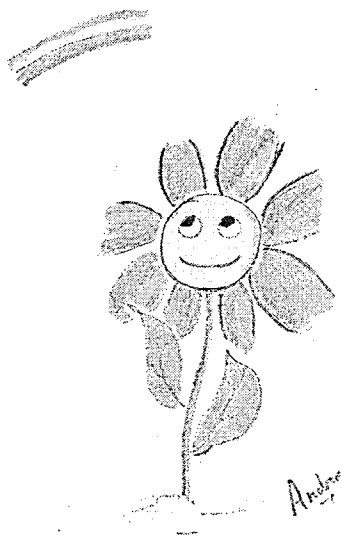
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'Mini had a happy day'